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Flavonoids with Novel Nicotinic Activity as Potential Pharmacotherapies to Treat Ethanol-Induced Neurotoxicity

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FLAVONOIDS WITH NOVEL NICOTINIC ACTIVITY AS POTENTIAL PHARMACOTHERAPIES TO TREAT ETHANOL-INDUCED NEUROTOXICITY

DISSERTATION

A dissertation in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Pharmacy at the University of Kentucky

By

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ABSTRACT OF DISSERTATION

FLAVONOIDS WITH NOVEL NICOTINIC ACTIVITY AS POTENTIAL PHARMACOTHERAPIES TO TREAT ETHANOL-INDUCED NEUROTOXICITY

Ethanol causes neurotoxicity via several mechanisms at different points in the cycle of dependence, including neuroinflammation and oxidative stress during ethanol exposure as well as excitotoxicity during ethanol withdrawal. The primary therapeutic implication is that ethanol-induced neurotoxicity requires multifunctional pharmacotherapies which reduce all mechanisms. Using an innovative pharmacological high throughput screening method on a large plant extract library we discovered flavonoids with alpha7 nicotinic acetylcholine receptor (nAChR) activity. In addition to their well-known anti-inflammatory and antioxidant properties, this novel activity means they can potentially reduce excitotoxicity and therefore makes them ideal for inhibition of ethanol-induced neurotoxicity. Rhamnetin, the candidate compound, was first found to inhibit lipopolysaccharide induced inflammation in immortalized BV2 microglia, in part, via alpha7 nAChRs. We then established an *in vitro* model of ethanol induced-neurotoxicity using organotypic hippocampal slice cultures which incorporated both neuroinflammatory and excitotoxic components. Neuroinflammation enhanced excitotoxicity under control conditions but the reverse was observed during ethanol withdrawal. Both mechanisms are important but their interaction is not simple. Finally, rhamnetin was evaluated in this model and found to reduce neuroinflammation and excitotoxicity associated with ethanol withdrawal. In conclusion, the studies herein provide strong evidence for alpha7 nAChRs selective flavonoids as potential pharmacotherapies for the treatment of ethanol-induced neurotoxicity and further implicate neuroinflammation, excitotoxicity, and their interaction as critical mechanisms in this pathology.

KEYWORDS: ethanol-induced neurotoxicity, neuroinflammation, excitotoxicity, alpha7 nicotinic acetylcholine receptor, rhamnetin

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CHAPTER 1. INTRODUCTION

1.1 General introduction

Alcohol dependence arises from a complex interaction of genetics, environment, and co-morbid psychiatric conditions and varies greatly between individuals in onset, course and duration. It results from the maladaptive and harmful consumption of alcohol ultimately leading to clinical impairment or distress as characterized by an increase in alcohol consumption, the persistent desire to consume alcohol, failure to cut down or control drinking, tolerance to the acute intoxicating effects of alcohol, and signs of alcohol withdrawal syndrome [1]. Alcohol dependence is estimated to affect 140 million people worldwide resulting in 3.3 million deaths associated with alcohol use every year [2]. In the USA, it is estimated to afflict 8.5% of the population [3] resulting in a massive medical, societal and economic burden [4].

Chronic alcohol use ultimately results in significant brain damage and loss as measured both post mortem [5] and *in vivo* using Magnetic Resonance Imaging (MRI) [6]. The majority of brain damage associated with alcohol dependence has been attributed to liver compromise and malnutrition, specifically thiamine deficiency, both of which contribute to the subsequent manifestation of Wernicke encephalopathy (WE) and Korsakoff syndrome (KS) [7]. Although WE and KS are well characterized neurological disorders with specific brain pathology and behavioral deficit, evidence of brain damage in uncomplicated alcoholics (no WE or KS) has also been shown in both post mortem and MRI studies [8]. Importantly, chronic alcohol consumption in uncomplicated alcoholics not only produces diffuse brain atrophy but also results in cognitive impairment [9], specifically memory and executive function deficits [10]. These behavioral deficits may, in turn, lead to excessive and chronic alcohol drinking. Therefore, alcohol-induced neurodegeneration has been hypothesized to contribute to

the progressive loss of control over drinking [11] and to be an integral component of the spiral of addiction [12]. In support, a recent prospective study in humans shows that volume deficits in specific brain regions were predictive of time to relapse into alcohol use [13].

Given the evidence for neurodegeneration in alcohol dependence, and the potential role of such cognitive impairment in promoting ongoing alcohol consumption, the development of treatment strategies to attenuate alcohol-induced neurodegeneration is imperative. Such a treatment would likely slow or even reverse cognitive impairment associated with alcohol use. This would allow an individual greater executive control over alcohol consumption and perhaps ultimately lead to recovery. However, with the possible exception of acamprosate (see below) there are no current neuroprotective pharmacotherapies approved for the treatment of alcohol dependence. Current FDA approved pharmacotherapies for alcohol dependence (i.e., disulfiram, naltrexone, and acamprosate) are primarily aimed at treating the behavioral manifestation of alcohol dependence. Specifically, disulfiram prevents the metabolism of acetaldehyde resulting in acute nefarious sensitivity to alcohol. Naltrexone is an opioid receptor antagonist which is thought to reduce the hedonic value of alcohol therefore reducing excessive drinking and the risk of relapse. Although the specific mechanisms of acamprosate are unknown, it is thought to reduce alcohol drinking by restoring balance to γ-aminobutyric acid (GABA) and glutamate neurotransmission. Importantly, acamprosate is also thought to reduce alcohol dependence in part due to its potential neuroprotective properties [14] providing initial support for the efficacy of neuroprotective drugs in the treatment of alcohol dependence.

In sum, alcohol dependence leads to harmful consumption of alcohol which results in significant brain damage and consequent cognitive deficits making it more

difficult to control alcohol consumption. As such, neuroprotective pharmacotherapies have been proposed as a treatment strategy for alcohol dependence and understanding the mechanisms of ethanol-induced neurotoxicity is essential for this aim. The following section will describe in detail specific mechanisms of ethanol-induced neurotoxicity, as well as their possible interactions.

1.2 Mechanisms of ethanol-induced neurotoxicity

Ethanol is a small organic molecule that is both hydrophilic and lipophilic, and can therefore easily pass through cell membranes and distribute readily throughout the entire body. Its small size and physicochemical properties also allow it to interact with a wide variety of biological substrates and ethanol therefore exhibits a wide spectrum of pharmacological activities. As such, ethanol has long been suspected to induce neurotoxicity through a variety of mechanisms which include, but are not limited to, oxidative stress, neuroinflammation and excitotoxicity. Importantly, these mechanisms are associated with different phases in the cycle of dependence. Ethanol exposure is associated with oxidative stress and neuroinflammation, whereas ethanol withdrawal (EWD) is associated with excitotoxicity. These mechanisms have been mostly studied individually as if they were separate but, in reality, they undoubtedly interact. Indeed, changes which occur during ethanol exposure are bound to extend into EWD and influence neurotoxicity at that time. The sections below will focus on mechanisms of ethanol-induced neurotoxicity during exposure and during EWD as well as their possible interactions.

1.2.1 Oxidative stress

Oxidative stress occurs in the majority of neurodegenerative diseases and manifests when the excessive generation of reactive oxygen species (ROS) surpasses endogenous antioxidant mechanisms [15]. In ethanol-induced neurotoxicity, oxidative

stress results primarily from the excessive generation of ROS as a consequence of ethanol metabolism [16]. The first step in the metabolism of ethanol is its conversion to acetaldehyde by alcohol dehydrogenase (ADH). Acetaldehyde is a highly reactive molecule which itself forms toxic protein adducts [17] and is in turn metabolized to acetate by acetaldehyde dehydrogenase (ALDH). Both metabolic steps rely on nicotinamide adenine dinucleotide (NAD) as a cofactor which is converted to its reduced form (NADH) in the process. The consequent increase in the $NADH:NAD^+$ ratio enhances the mitochondrial electron transport chain which leads to the formation of ROS [18]. Excessive ROS can freely react with DNA, proteins and lipids resulting in direct structural DNA damage, protein dysfunction and lipid peroxidation, culminating in cell death. This has important implications for peripheral tissue damage, particularly in the liver, where the majority of ADH and ALDH are found. However, ethanol induces the expression of ROS generating cytochrome P450 2E1 (CYP2E1) both in the liver [19] and in the brain [20]. In the brain, CYP2E1 is capable of metabolizing ethanol which generates ROS [21]. Therefore, although oxidative stress is particularly relevant for alcohol liver disease it is also important as a toxic mechanism in the brain. In support, potent antioxidants, such as ascorbic acid, thiamine, quercetin [22], cyanidin-3-glucoside [23], cannabidoil, butylated hydroxytoluene (BHT), alpha-tocopherol [24, 25] and tocotrienol [26] decrease oxidative stress and neurotoxicity in various models of ethanol-induced neurotoxicity. In sum, ethanol metabolism generates oxidative stress in all cells in which it occurs. Therefore, this mechanism of neurotoxicity is particularly relevant during ethanol exposure.

1.2.2 Neuroinflammation

Neuroinflammation is thought to contribute to a variety of neurodegenerative diseases [27] and to a variety of acute neuronal injuries such as stroke [28] and

traumatic brain injury [29]. As such, neuroinflammation has also been proposed as a potential mechanism of neurotoxicity during ethanol exposure. In fact, research on peripheral tissues has long recognized chronic inflammation as an important factor in ethanol-induced tissue damage. Chronic ethanol consumption increases the leakiness of the gut which results in elevated levels of circulating bacterial endotoxin, lipopolysaccharide (LPS) [30]. In turn, LPS activates peripheral immune cells which release toxic proinflammatory cytokines. Since ethanol disrupts the blood brain barrier (BBB) [31], LPS may enter the central nervous system (CNS) and activate neuroimmune cells despite its low permeability under normal physiological conditions [32]. As such, postmortem human alcoholic brains exhibit upregulated neuroimmune gene expression [33] and increased markers of microglial activation and proinflammatory signaling [34]. However, it is still unclear whether this is a result of LPS infiltration in the brain or the direct effects of ethanol on neuroimmune cells. Indeed, studies *in vitro* show that ethanol directly activates toll-like receptor 4 (TLR4) in a variety of isolated neuroimmune cells [35, 36]. TLR4 is the main receptor for the innate immune system and also the primary target for LPS. These studies were extended to isolated microglia where ethanol activates TLR4 and induces a proinflammatory response [37, 38]. Moreover, these *in vitro* studies were corroborated *in vivo* using TLR4 knock-out mice [39]. Interestingly, mice chronically force-fed ethanol for 10 days exhibited increased proinflammatory signaling in the brain and, more importantly, a sensitized proinflammatory response to a subsequent challenge with LPS [40]. Thus, ethanol and increased LPS permeability may both be responsible for the excessive proinflammatory signaling observed in the brain during ethanol exposure.

When neuroinflammatory signaling becomes chronic and/or excessive it can induce neurotoxicity through a variety of mechanisms, including apoptosis, excitotoxicity,

immune activation and oxidative stress [41]. This already suggests significant interactions with other mechanisms of ethanol-induced neurotoxicity. However, evidence for the direct contribution of neuroinflammation to ethanol-induced neurotoxicity is limited and usually involves other mechanisms, such as oxidative stress. For example, curcumin [42] and cyanidin-3-glucoside [23], have both been shown to reduce neuroinflammation and neuronal damage in rodent neonatal models of ethanol-induced neurotoxicity. However, both compounds are known antioxidants [43, 44] and thus their anti-inflammatory effects are not necessarily responsible for their neuroprotective effects. Similarly, using the Majchrowicz binge ethanol protocol [45], BHT protects against ethanol induced brain damage presumably through anti-inflammatory effects as it was found to reduce NF-KappaB binding [25]. However, BHT is also known to have potent antioxidant properties. The most compelling evidence for the direct contribution of neuroinflammation in ethanol-induced neurotoxicity so far is from studies using TLR4 knockout mice, which are protected against ethanol-induced neuronal damage [39].

Taken together, ethanol induces proinflammatory signaling during ethanol exposure and may cause neurotoxicity at that time. This could be due to the direct effects of ethanol on neuroimmune cells and/or the infiltration of LPS into the brain through compromised gut and BBB. The combination of these mechanisms is more likely but this remains uncertain today. Nevertheless, neuroinflammation is therefore a mechanism of neurotoxicity that is particularly relevant during ethanol exposure, but given its downstream effects it is likely to affect excitotoxicity during EWD. The work presented in this dissertation aims to shed some light on this particular interaction.

1.2.3 Excitotoxicity

Ethanol has profound effects on the glutamate system in the CNS, and EWD-induced excitotoxicity is believed to result from maladaptive alterations to this system

that occur during ethanol exposure. Ethanol exposure upregulates the number of N-methyl-D-aspartate receptors (NMDAR) [46, 47] and/or changes their subunit composition [48], resulting in hypersensitive conformations of the receptor [49]. This is presumed to occur via homeostatic changes in response to the acute inhibitory effects of ethanol at the NMDAR [50]. In parallel, several lines of investigation have found that release of glutamate [51] and polyamines [52] is greater during EWD, potentially compounding the effects of functionally up-regulated NMDARs. The overall result is the excessive activation of already sensitive NMDARs and the consequent influx of excessive calcium (Ca^{2+}) into neurons during EWD. Indeed, extensive work from our laboratory using organotypic rat hippocampal cultures chronically exposed to ethanol supports these mechanisms. As such, we have shown that ethanol exposure induces glutamate, putrescine and spermidine release in culture media during EWD [52] and increases polypeptide expression of mGluR5 and NR1 and NR2B subunits of NMDARs [53]. Moreover, EWD induced cytotoxicity is enhanced by co-exposure to spermidine [54] and EWD induced Ca^{2+} can be blocked by NMDAR antagonists [55]. In turn, Prendergast et al showed in the same preparations that rapid and significant Ca^{2+} entry occurs during EWD which is followed by excitotoxic cell death [56] and that prolonged ethanol exposure was found to significantly reduce immunolabeling of calbindin-D_{28k}, a calcium buffering protein, suggesting that the neuronal capacity to buffer excessive Ca^{2+} is also impaired [57]. Aberrant intracellular Ca^{2+} has, in turn, been proposed to activate Ca^{2+} dependent lytic enzymes, disrupt mitochondrial function ultimately leading to ROS generation and neuronal damage [58, 59].

In support, several *in vitro* studies using NMDAR antagonists with subtly different mechanisms show that blocking these receptors prevents excessive intracellular Ca^{2+} during EWD and consequent neurotoxicity. For example, MK-801 and ifenprodil inhibit

neurotoxicity and Ca^{2+} entry induced by EWD in organotypic hippocampal slices [55]. Similarly, memantine was shown to reduce EWD induced neurotoxicity *in vitro* [60]. Moreover, several *in vivo* studies have shown that NMDAR antagonists can reduce seizure activity associated with EWD and the consequent cognitive deficits. As such, memantine reduces EWD induced convulsions [60], MK-801 blocks EWD seizures [61] and 4 weeks of memantine treatment during EWD completely reverses cognitive deficits, as measured by the Morris water maze, 10 weeks after the onset EWD [62].

Taken together, ethanol exposure produces profound neuroadaptations to the glutamate system ultimately resulting in hyperexcitable state which leads to excitotoxicity when ethanol is removed. Therefore, this mechanism of neurotoxicity is primarily relevant to periods of EWD.

1.3 Possible interactions

Clinically, alcohol dependence is a chronic relapsing condition in which repeated bouts of ethanol exposure and EWD become cyclical. Therefore, changes occurring during ethanol exposure may have important implications for mechanisms of neurotoxicity during EWD. Conversely, changes occurring during EWD may also have important implications for the mechanisms of neurotoxicity during subsequent ethanol exposure.

1.3.1 Changes during ethanol exposure may affect neurotoxic mechanisms during EWD

Although generation of ROS is primarily a consequence of ethanol metabolism, neuroinflammation also leads to ROS generation [63, 64]. Therefore, during ethanol exposure, ethanol metabolism and neuroinflammation may act hand in hand to produce excessive amounts of ROS. Since excitotoxic injuries also involve the generation of ROS

[65], the impact of ROS generation during exposure may extend into EWD and contribute to excitotoxicity.

Changes to neuroimmune signaling induced by ethanol may also have important implications for excitotoxicity during EWD. In fact, neuroinflammation has been shown to exacerbate excitotoxicity [66] through a variety of mechanisms. For example, activated microglia release quinolinic acid [67] and glutamate [68], which are both capable of activating the NMDAR. TNF-alpha, one of the main proinflammatory cytokines, downregulates EAAT2/GLT1 expression on glia [69], in turn increasing extra-synaptic glutamate concentrations. TNF-alpha has also been found to upregulate Ca^{2+} permeable AMPA receptors [70, 71] which may contribute to overall glutamate hyperexcitability. Similarly, nitric oxide (NO), another proinflammatory mediator, directly contributes to excitotoxicity [72-74] and induces glutamate release from neurons by inhibiting neuronal respiration [75]. Therefore, changes in neuroimmune signaling, which tend to be prolonged, initiated during ethanol exposure may extend into EWD and enhance excitotoxicity.

The fact that specific mechanisms initiated during ethanol exposure may contribute to or enhance neurotoxic mechanisms during EWD does not exclude the fact that the opposite may also be true and adaptive changes during ethanol exposure may help reduce toxicity during EWD. For example, increased levels of ROS scavenger enzymes, including superoxide dismutase and glutathione reductase, have been observed in erythrocytes from alcoholic individuals [76]. If similar changes occur in the brain this could reduce the ROS-mediated consequences of excitotoxicity in AWD. However, evidence for neuroprotective adaptations induced by ethanol exposure is limited.

1.3.2 Changes during EWD may affect neurotoxic mechanisms during subsequent ethanol exposure

It is clear that changes in oxidative stress and neuroinflammation during ethanol exposure are likely to affect excitotoxicity during EWD. However, there is limited evidence on the effects of excitotoxicity on oxidative stress and neuroinflammation associated with a subsequent exposure to ethanol. This is surprising given the fact that EWD is well known to have a kindling effect and repeated EWDs increase in severity [77, 78]. Nevertheless, there is some evidence that excitotoxicity may enhance oxidative stress and neuroinflammation. As mentioned above, excitotoxicity involves the generation of ROS [65] and may add to the pool of reactive species when ethanol metabolism is resumed during subsequent ethanol exposure. Additionally, excitotoxicity has been shown to induce a delayed proinflammatory response [79] which may affect subsequent neuroinflammatory signaling induced by ethanol exposure. The research presented in this dissertation focuses primarily on the impact of ethanol exposure during EWD and not on the impact of EWD on subsequent exposure. However, repeated EWD paradigms, such as *in vivo* intermittent access to ethanol [80] or *in vitro* repeated ethanol exposure using organotypic cultures [81], may be valuable for evaluating the effects of EWD-induced excitotoxicity on oxidative stress and neuroinflammation during subsequent ethanol exposure.

1.4 Therapeutic implications

As described in the previous section, ethanol causes neurotoxicity via several mechanisms at different points in the cycle of dependence and these mechanisms undoubtedly interact. Therefore, the primary therapeutic implication is that alcohol-induced neurodegeneration requires multi-functional pharmacotherapies targeted at numerous mechanisms of ethanol-induced neurotoxicity. This has already been

recognized for other neurodegenerative diseases, such as Alzheimer's [82] and Parkinson's disease [83], for which multi-targeted designed drugs are currently being developed [84]. Multi-targeted designed drugs entail a single pharmacotherapy that is either pharmacologically promiscuous enough to hit numerous targets simultaneously, or one that is specific to a single target that is capable of interfering with several pathological mechanisms. One such single target is the alpha7 nicotinic acetylcholine receptor which has emerged as a pluripotent target for the treatment of neurodegenerative diseases [85].

Nicotinic acetylcholine receptors (nAChRs) form a large heterogeneous family of ligand gated cationic receptors by combining 12 different subunits (alpha2-alpha10, beta2-beta4) into heteromeric and homomeric pentamers. They are widely and differentially expressed in various regions throughout the CNS and serve a variety of functions. The major subtypes found in the brain are the alpha4-beta2 heteromeric nAChRs and the alpha7 homomeric nAChRs. Alpha7 nAChRs are discreetly distributed throughout the CNS and are mainly found in hippocampal, hypothalamic and cerebellar neurons as well as the 10th cranial nerve, the vagus (Latin "wanderer"). Moreover, alpha7 nAChRs have been found on non-neuronal cells such as macrophages [86], astrocytes [87], and microglia [88]. Thus, alpha7 nAChRs are present on neurons and neuroimmune cells and the following sections will discuss how their activation has the potential to attenuate neuroinflammation and excitotoxicity.

1.4.1 Alpha7 nAChR activation on microglia reduces neuroinflammation

The potential role of nAChRs in immune regulation was first described with the discovery of the cholinergic anti-inflammatory pathway which involves the vagus nerve and its major neurotransmitter, acetylcholine. As such, acetylcholine has been shown to inhibit LPS-induced release of proinflammatory cytokines from peripheral macrophages,

an effect that could be blocked by alpha-conotoxin [89]. In the same study, the authors were able to show that vagal nerve stimulation could rescue animals from a lethal dose of LPS, thereby implicating both acetylcholine and the vagus nerve in protection against sepsis. Subsequent studies showed that the endogenous target for the anti-inflammatory effects of acetylcholine was the alpha7 nAChR making it central in neurological control of peripheral inflammation [90]. For example, Wang et al showed in an elegant study using alpha7 nAChR specific antisense oligonucleotides, that nicotine inhibits LPS-induced TNF synthesis in macrophage cultures specifically via alpha7 nAChRs [86]. The pivotal role that nAChRs play in control of inflammation may in turn explain the negative association of cigarette smoking and the incidence of inflammatory diseases, such as ulcerative colitis [91]. Alpha7 nAChRs were then found to not only be important in peripheral inflammatory processes but also be important in inflammatory processes in the brain. Indeed, alpha7 nAChRs are expressed on microglia and nicotine or acetylcholine dose dependently attenuate microglial activation as well as LPS-induced TNF-alpha release through an alpha7 nAChR dependent pathway [88]. A number of intracellular signaling pathways have been implicated in alpha7 nAChR inflammatory modulation, namely reduction of nuclear factor kappa-B (NF-KB) mediated transcription [92], inhibition of mitogen-activated protein kinase (MAPK) phosphorylation [88] and phosphorylation of protein kinase B (Akt) through phophatidyl-4,5-bisphosphate 3-kinase (PI3K) activation [93], but the specific mechanisms remain unclear. Taken together, alpha7 nAChRs are central to inflammatory signaling in both the periphery and in the CNS and are therefore valuable as pharmacological targets for reducing neuroinflammation [94].

1.4.2 Alpha7 nAChR activation on neurons reduces excitotoxicity

The neuroprotective properties of nicotine have been extensively studied in the context of neurodegenerative diseases and was probably sparked, in part, by the negative association of cigarette smoking with the incidence of AD and PD [95]. As such, nicotine has been shown to be neuroprotective in a variety of *in vitro* and *in vivo* models of neurodegenerative diseases and brain injury (reviewed elsewhere [96]). Specifically, nicotinic agonists have been extensively shown to protect neurons from excitotoxic injury. For example, the original studies by Akaike et al show that nicotine protects cultured cortical neurons against glutamate toxicity [97]. Subsequently, nicotine was shown to prevent NMDA-induced toxicity in primary hippocampal neurons specifically via alpha7 nAChR activation because the neuroprotective effects of nicotine were blocked by methyl-lycaconitine (MLA) [98]. Moreover, similar findings were shown in hippocampal slice cultures [99]. Eventually, the neuroprotective effects of nicotine were shown to be effective against ethanol-induced excitotoxicity in that nicotine exposure attenuates EWD induced neurotoxicity in hippocampal explants [100]. In support, 3-[2,4-dimethoxybenzylidene]anabaseine (DMXB), a relatively selective agonist for the alpha7 nAChR, was also shown to inhibit ethanol induced neurotoxicity [101-104]. The specific downstream mechanisms responsible for the neuroprotection afforded by alpha7 nAChR activation are still uncertain but a few possibilities have been proposed. For example, increased intracellular Ca^{2+} buffering has been proposed as a potential mechanism because nicotine treatment significantly increased calbindin-D_{28k} immunoreactivity in hippocampal slice cultures [57]. Another possibility is via NMDAR cross-desensitization. For example, donepezil hydrochloride, which directly modulates nAChR [105], was found to mediate phosphorylation and subsequent internalization of NMDAR subunits NR1 and NR2A via alpha7 nAChR activation thereby preventing excitotoxicity [106]. Taken

together, alpha7 nAChRs constitute a viable pharmacological target to reduce excitotoxicity associated with EWD. In addition, emerging preclinical and clinical studies suggests that the cholinergic system may also be a valuable target for reducing ethanol drinking behaviors [107].

1.5 Finding novel alpha7 nAChR selective compounds in plants

As described in the previous section, alpha7 nAChR selective agonists constitute potential pharmacotherapies for the treatment of neurodegenerative diseases by attenuating neuroinflammation and excitotoxicity. In fact, there have been considerable efforts made towards synthesizing alpha7 nAChR selective ligands in the last decade [108] but none have made it to market. Interestingly, most of these synthetic compounds are derived from alkaloids originally discovered in plants [109]. Most plant nAChR ligands are simple alkaloids like nicotine, anatabine, and lobeline which are either toxic, or have abuse liability, due partly to their high affinity and selectivity for alpha4-beta2 nAChRs [110]. However, not all plant alkaloids have this selectivity and MLA, for example, is a relatively selective antagonist for alpha7 nAChRs [111, 112]. This suggests that plants may contain novel alpha7 nAChR selective ligands that would have therapeutic value for the treatment of neurodegenerative diseases.

Traditional approaches to plant drug discovery are time consuming and costly because they usually require separation and purification of compounds before pharmacological evaluation. Moreover, using a single pharmacological screen on plant extracts would likely yield extremely high hit rates making it difficult to prioritize one plant extract over another. However, applying several pharmacological screens to plant extracts circumvents these difficulties and can yield lower hit rates. Thus, to identify plants containing alpha7 selective compounds we developed a differential smart screening approach that relies on three individual pharmacological screens and applied

it to a large plant extract library [113]. In brief, plant extracts were first evaluated for their ability to displace a nonsubtype selective nAChR ligand, [^{3}H]-epibatidine, in rat brain homogenates to assess the presence of any nAChR activity. Plant extracts that displayed nAChR activity were then evaluated and compared simultaneously for their ability to displace an alpha4-beta2 selective ligand, [^{3}H]-cytisine, and an alpha7 selective ligand, [^{3}H]-MLA. Plant extracts that displaced [3H]-MLA at lower concentrations than they displaced [3H]-cytisine were considered likely to contain alpha7-selective compounds. Only 8 species displayed this pharmacological signature and *Solidago nemoralis* was chosen for further investigation because this genus has not been reported to contain bioactive alkaloids (making it likely that active compounds would be novel). Identifying and characterizing the compounds responsible for this activity is a primary aim of the current dissertation.

1.6 Project overview

This dissertation focuses on the role of neuroinflammation in modulating excitotoxicity which occurs during EWD. The primary hypothesis is that enhanced neuroinflammation associated with ethanol exposure potentiates excitotoxicity during EWD. The primary therapeutic implication is therefore that ethanol neurotoxicity requires multi-functional pharmacotherapies that reduce neuroinflammation and excitotoxicity. Alpha7 nAChR selective pharmacotherapies have the potential to do both and novel compounds from *Solidago nemoralis* with relative selectivity for this receptor subtype were investigated in the work herein.

The aims of this dissertation are to (1) increase our understanding of the interaction between neuroinflammation and excitotoxicity during EWD, (2) identify and characterize novel natural products with alpha7 nAChR selective activity and (3) evaluate their anti-inflammatory and neuroprotective properties *in vitro*. Thus, the first

chapter explains how we developed an *in vitro* model of ethanol induced neurotoxicity which includes neuroinflammatory and excitotoxic components, the second chapter presents the discovery of specific flavonoids with selectivity for alpha7 nAChRs relative to alpha4-beta2 and how this activity may contribute to their well-known anti-inflammatory properties, and in the third chapter we evaluate the candidate flavonoid rhamnetin in the model of ethanol induced neurotoxicity described in chapter 1.

- Primary hypothesis: enhanced neuroinflammation associated with ethanol exposure potentiates excitotoxicity during EWD
- Therapeutic implication: ethanol-induced neurotoxicity requires multi-functional pharmacotherapies targeted at reducing neuroinflammation and excitotoxicity
- Therapeutic target: alpha7 nAChR agonists have the potential to reduce neuroinflammation and excitotoxicity
- Therapeutic aim: to identify and characterize novel alpha7 nAChR selective plant natural products

CHAPTER 2. ALTERED RELATION BETWEEN LIPOPOLYSACCHARIDE-INDUCED INFLAMMATORY RESPONSE AND EXCITOTOXICITY IN RAT ORGANOTYPIC HIPPOCAMPAL SLICE CULTURES DURING ETHANOL WITHDRAWAL

Manuscript submitted to Alcoholism: Clinical and Experimental Research

2.1 Introduction

Ethanol causes neurotoxicity via several mechanisms [114] at different points in the cycle of dependence, including ethanol exposure and ethanol withdrawal (EWD). Ethanol exposure is generally associated more with oxidative stress [64] and neuroinflammation [115] whereas EWD is associated with excitotoxicity [59]. These mechanisms have mostly been studied as if they were separate, but the possibility that there are important interactions between them has rarely been considered. For example, although oxidative stress is most directly a consequence of ethanol metabolism [116], the generation of reactive oxygen species is also part of the mechanism of toxicity in excitotoxic damage [117]. Therefore, the impact of ethanol metabolism can extend into EWD and contribute to oxidative stress produced by excitotoxicity. Similarly, neuroimmune signaling changes induced by ethanol exposure may extend into EWD and potentiate excitotoxicity observed at that time. In this study we investigate the potential interaction between neuroinflammation and excitotoxicity during EWD. A major aim is to identify cellular and molecular targets in order to devise novel pharmacotherapies for ethanol-induced neurotoxicity.

The precise mechanisms that cause excitotoxicity during EWD are uncertain, but increased activity of the glutamate system is fundamental [118]. Most evidence supports two possible mechanisms that are not exclusionary. One is that ethanol exposure upregulates the number of N-methyl-D-aspartate receptors (NMDAR) [47] and/or changes their subunit composition [48], resulting in hypersensitive conformations of the

receptor [49]. The second is that release of glutamate [51] and polyamines [52] is greater during EWD, resulting in the overactivation of NMDARs. These mechanisms of EWD-induced excitotoxicity are closely interrelated *in vivo*, but they can be evaluated separately ex vivo in cell culture systems such as organotypic hippocampal slice cultures (OHSC) [119] where exogenous activation of NMDARs can be used to cause neurotoxicity. Studies on OHSC (e.g. [52, 53]) suggest that both the functional upregulation of NMDARs and increased release of glutamate and polyamines contribute to EWD-induced excitotoxicity in this model. In the current study we focused on NMDAR hypersensitivity by activating these receptors with exogenous NMDA during EWD. Many previous studies (e.g. [55, 120]) have shown that NMDA-induced toxicity is enhanced under EWD conditions.

None of the above studies exclude other mechanisms, such as neuroinflammation, as contributing to excitotoxicity during EWD. In support, it has previously been reported that neuroinflammation enhances excitotoxicity in OHSC [121]. Therefore, the current study tested the primary hypothesis that ethanol exposure causes neuroinflammation, which then potentiates excitotoxicity during EWD.

To date, research on ethanol exposure and neuroinflammation has focused on changes that occur in the presence of ethanol rather than during EWD. Increased release of proinflammatory mediators from microglia, the primary immune cells of the central nervous system, as a consequence of ethanol exposure is probably fundamental, but as with excitotoxicity, two major mechanisms have been proposed for this. One is that ethanol exposure results in increased influx of lipopolysaccharide (LPS), a bacterial endotoxin, via the gut [30] and blood brain barrier [31], thereby activating microglia directly. The second is that ethanol exposure directly affects microglia [38] and sensitizes their response to LPS [40]. The possible contribution of these mechanisms is

difficult to separate *in vivo* but, as with excitotoxicity, OHSC can be used to dissect these possibilities because exogenous LPS can be used to activate microglia in the culture [122].

To test the primary hypothesis, OHSCs were stimulated to release proinflammatory mediators by treatment with exogenous LPS during EWD. If prior ethanol exposure sensitizes microglia, OHSC should release greater amounts of proinflammatory mediators in response to LPS during EWD. In turn, the enhanced proinflammatory response should lead to a further enhancement of excitotoxicity induced by an NMDA challenge. The specific prediction to be tested in these experiments is therefore that ethanol exposure should result in increased excitotoxicity when OHSC are challenged with both LPS and NMDA during EWD. Previous studies have used OHSC to study the effects of ethanol on excitotoxicity [123] and neuroinflammation [81] but there are no published studies on the interaction between these.

Microglia have a fundamental role in the hypotheses that connect ethanol-induced neurotoxicity with neuroinflammation [124]. However, all brain preparations, including OHSCs, contain other types of glia, including astrocytes [125] and oligodendrocytes [126] as well as microglia [127]. All these cell types may contribute to the LPS-induced inflammatory response [128] and modify excitotoxicity. In an attempt to address this issue we evaluated the effects of chronic ethanol exposure on cultures in which only immortalized microglia of the BV2 cell line are present. We then evaluated their subsequent response to LPS during EWD, thereby mirroring the studies on OHSC. If ethanol induces direct effects specifically on microglia, and these effects are responsible for changes observed in OHSCs, then it should be possible to observe similar effects in BV2 cell cultures.

Together these studies aim to increase our understanding of the mechanisms underlying excitotoxicity associated with EWD. It should be emphasized that OHSC and immortalized BV2 microglia are being used to dissect mechanisms of ethanol-induced neurotoxicity rather than to “model” all aspects of alcohol-induced neurodegeneration. Subsequent studies *in vivo* will be necessary to confirm the observations *in vitro*, but the culture systems provide a means of identifying specific cell types and molecular targets that are of potential value in treating this aspect of alcoholism.

2.2 Materials and methods

2.2.1 Organotypic hippocampal slice culture (OHSC) preparation

OHSC were prepared essentially as described by Stoppini et al. [129]. Briefly, hippocampi were aseptically removed from 8-day-old Sprague-Dawley male and female rat pups and sliced at a transverse thickness of 200 µm using a McIlwain tissue chopper (Campden Instruments Ltd., Lafayette, ID). Slices were transferred to sterile culture inserts (4 slices per insert) and placed in 6-well-plates containing culture medium (Minimum Essential Medium (Life Technologies Corporation, Grand Island, NY), 200mM glutamine (Invitrogen, Carlsbad, CA), 25mM HEPES (ATCC, Manassas, VA), 50uM penicillin/streptomycin (ATCC, Manassas, VA), 36mM glucose, 25% (v/v) Hank’s buffered salt solution (Gibco BRL, Gaithersburg, MD), 25% heat-inactivated horse serum (Sigma, St. Louis, MO)). Cultures were maintained at 37°C in an atmosphere of 5% CO2/95% air in 95% humidity for 5 days *in vitro* (DIV) to allow slices to adhere to the insert membrane. The care of animals was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996), as well as the University of Kentucky’s Institutional Animal Care and Use Committee.

2.2.2 Ethanol exposure and withdrawal (EWD)

At 6 DIV, slices were placed in culture media with or without the addition of 100mM ethanol, incubated for 10 DIV and then subjected to EWD for 24h, 48h or 72h. During exposure, the plates were placed in topless polypropylene containers containing 50mL of 100mM ethanol or dH2O accordingly, and placed inside sealable plastic bags filled with 5% CO2, 21% oxygen, and a balance of nitrogen. The ethanol solution was used as an evaporating source of ethanol to counter the evaporation of ethanol from the culture wells resulting in an average ethanol concentration of 65mM (a concentration that approximates 300mg/dL) during the 5 DIV between media changes. At the onset of EWD, slices were further separated into groups and treated accordingly: control media, 5uM NMDA (Sigma Aldrich Co. LCC., St. Louis, MO), 10ug/mL lipopolysaccharide from *Escherichia coli* 026:B6 (LPS, Lot #: 021M4072V; Sigma Aldrich Co. LCC., St. Louis, MO) or both NMDA and LPS combined. A single concentration of LPS was used in these experiments because in preliminary experiments using a range of concentrations (100ng/mL, 1ug/ml and 10ug/mL) 10ug/mL produced measurable release of TNFalpha and nitric oxide. Similarly, a single concentration of NMDA was used because previous work from our laboratory [52] shows that 5uM NMDA is the lowest concentration to consistently produce significant toxicity in OHSC.

2.2.3 Assessment of toxicity by propidium iodide uptake

Propidium iodide (PI) is a membrane impermeable, DNA intercalating fluorescent molecule that is commonly used in OSHC as a semi-quantitative stain for cellular toxicity and has been significantly correlated to other reliable markers of cell death [130]. Although there has been extensive work on NMDA and EWD toxicity in OHSC using a variety of cell-type specific markers [131], it is uncertain how these markers are affected

by LPS in this model. The current study focused on the interaction between NMDA and LPS and since PI uptake has been used in OHSC to assess both NMDA [131] and LPS [122] induced toxicity separately, it was therefore chosen for the current study to assess overall toxicity.

For 24h EWD, slices were directly challenged in culture media containing 3.74uM propidium iodide (PI; Sigma Aldrich Co. LCC., St. Louis, MO). For 48h and 72h EWD, slices were originally challenged in culture media without PI and each well was supplemented with 10uL of concentrated PI (374uM) to obtain a final concentration of 3.74uM PI 24h prior to imaging. Slice images were captured using SPOT Advanced software (Version 4.0.9; W. Nuhsbaum Inc., McHenry, IL) connected to an inverted Leica DMIRB microscope (W. Nuhsbaum Inc.) fitted for fluorescence detection (mercury-arc lamp) and connected to a computer via a SPOT 7.2 color mosaic camera (W. Nuhsbaum Inc). PI uptake in the CA1, CA3, and DG cell layers was measured using ImageJ software (Version 1.46; National Institute of Health, Bethesda, MD). Background signal was subtracted from intensities obtained for each cell layer resulting in specific intensities which were used for statistical analysis. These values were then converted to % control (no EWD, no NMDA, and no LPS) within each preparation for graphical representation and clarity across time points.

2.2.4 Assessment of inflammatory mediator release

Once slices were imaged, inserts were discarded and the resulting media was collected for assessment of inflammatory mediator release. Nitric oxide (NO) release was assessed by the Griess Reagent System (Promega Corporation, Madison, WI) according to the manufacturer's instructions. All samples were assayed in duplicate and nitrite content was estimated using a reference $NaNO_2$ standard curve performed with each assay. TNF-alpha content was assessed by enzyme linked immunosorbent assay

kit (ELISA; Ready-Set-Go!® ELISA, eBioscience Inc., San Diego, CA) according to the manufacturer's instructions. All samples were assayed in duplicate and TNF-alpha content was estimated from a reference TNF-alpha standard curve performed with each assay.

2.2.5 BV2 microglia culture

BV2 microglia are derived raf/myc-immortalized murine neonatal microglia. They were cultured in Dulbecco's Modified Eagle Medium/Ham's F12 nutrient mix supplemented with 10% fetal bovine serum and antibiotics (penicillin 100U/ml, streptomycin 100ug/ml; all from Life Technologies Corp., Grand Island, NY). Cells were kept at 37°C in a humidified atmosphere of air and 5% CO_2 and propagated in T25 flasks (Techno Plastic Products AG, Trasadingen, Switzerland) in media with or without 100mM EtOH in sealable plastic bags as described above to avoid EtOH evaporation. They were split every 2-3 days and subjected to EWD after 10 days. During EWD, cells were seeded in 24 well plates at densities of $5x10^5$ cells/well, allowed to adhere overnight and then challenged with 1µg/mL LPS for 24h. Culture media was collected and assessed for inflammatory mediators as described above. Cell viability was measured using resazurin salt fluorescence (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt; Sigma Aldrich Co. LCC., St. Louis, MO) and normalized to percentage control (no LPS group).

2.2.6 Statistical analysis

Data were analyzed using IBM Statistical Package for the Social Sciences (SPSS) Version 21 (IBM Corporation, Armonk, NY) and graphed using Prism (Graphpad Software Inc., La Jolla, CA). PI uptake was measured in three different regions (DG, CA3 and CA1). Thus, PI uptake was analyzed by multi-factorial, repeated measures analysis of variance (ANOVA) with region as within-subjects variables and sex, EWD,

NMDA, and LPS as between-subjects factors. Data were obtained from separate preparations for each length of treatment and EWD (24h, 48h, and 72h). Thus, preparation and time point were used as covariates to control for differences across litters/culture preparations and time of exposure. Significant interactions were further investigated at each time point using post hoc pair-wise comparisons using Fisher's LSD test. TNF-alpha and NO release from OHSC were also analyzed by multi-factorial ANOVA with the following factors: sex, EWD, NMDA and LPS as well as preparation and time point as covariates. Significant interactions were further investigated at each time point using post hoc pair-wise comparisons with Tukey's LSD correction. TNF-alpha and NO release from BV2 microglia were analyzed by non-repeated measures two-way ANOVA with EWD and LPS as factors followed by Bonferroni post-hoc analyses.

2.3 Results

2.3.1 Effects of NMDA, LPS and EWD on cellular damage in OHSC as measured by PI uptake

An overall repeated-measures multi-factorial ANOVA was first performed on PI uptake across the treatment groups to assess whether there were any differences in the separate regions of the hippocampus and whether there were any sex effects. This analysis revealed that sex did not interact with any factors but that there was an effect of region and the highest order significant interaction included all factors except for sex (region x EWD x NMDA x LPS [$F(1.09,1765.03) = 15.02$, $p < 0.0001$] corrected using Greenhouse-Geisser). Therefore, subsequent analyses were performed within each region at each time point collapsed across sex. Analysis within each region revealed minor effects of drug treatment on PI uptake in the DG and CA3 in comparison to those observed in the CA1. Therefore, assessment of cellular damage by PI uptake was focused on the CA1 region of the hippocampus.

2.3.1.1 LPS treatment potentiates NMDA-induced cellular damage under control conditions

Before we could evaluate the combined effects of NMDA and LPS on hippocampal damage, we examined the effects of LPS alone under control conditions and during EWD. Slices treated with LPS alone under both conditions exhibited a speckled pattern of PI uptake throughout the slice that was not observed in untreated slices (fig 2.1). However, when quantified in the CA1 region of the hippocampus, it did not differ from untreated control or EWD slices (fig. 2.2). Despite no quantifiable effect alone, LPS significantly potentiated NMDA-induced damage under control conditions. Slices co-exposed to LPS and NMDA exhibited greater PI uptake at all time points in comparison to slices treated with NMDA alone (24h = 279%, post hoc [$p < 0.01$], 48h = 275%, post hoc [$p < 0.0001$] and 72h = 201%, post hoc [$p < 0.0001$], all compared to NMDA alone).

2.3.1.2 EWD potentiates peak NMDA-induced cellular damage

OHSC treated with NMDA under control conditions exhibited significant cellular damage in the CA1 region of the hippocampus as measured by PI uptake (fig. 2.2 - 222%, post hoc [$p < 0.0001$] compared to control). When coupled with EWD, PI uptake was significantly potentiated at the 24h time point (363%, post hoc [$p < 0.0001$] compared to NMDA). At later time points (48h and 72h), PI uptake for these groups remained significantly higher than control (post hoc [$p < 0.05$] compared to control at 48h and 72h) but they were not statistically different from each other.

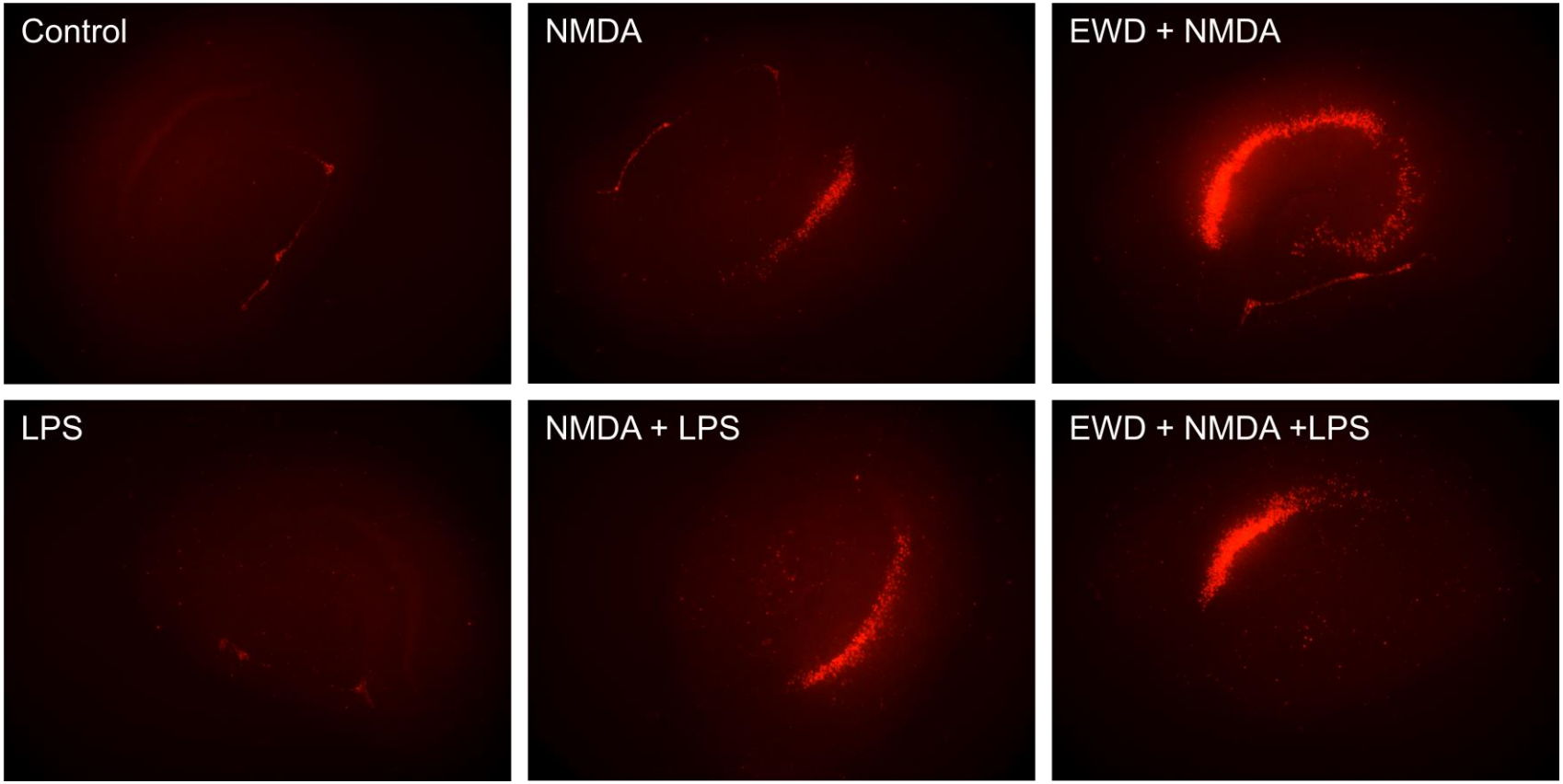

Figure 2.1 Lipopolysaccharide (LPS) potentiates N-methyl-D-aspartate (NMDA) toxicity under control conditions and reduces NMDA toxicity during ethanol withdrawal (EWD)

Representative images of propidium iodide uptake in organotypic hippocampal slice cultures left untreated (control) or treated with LPS, NMDA or a combination of both (NMDA+LPS) for 24h under control conditions and under EWD conditions.

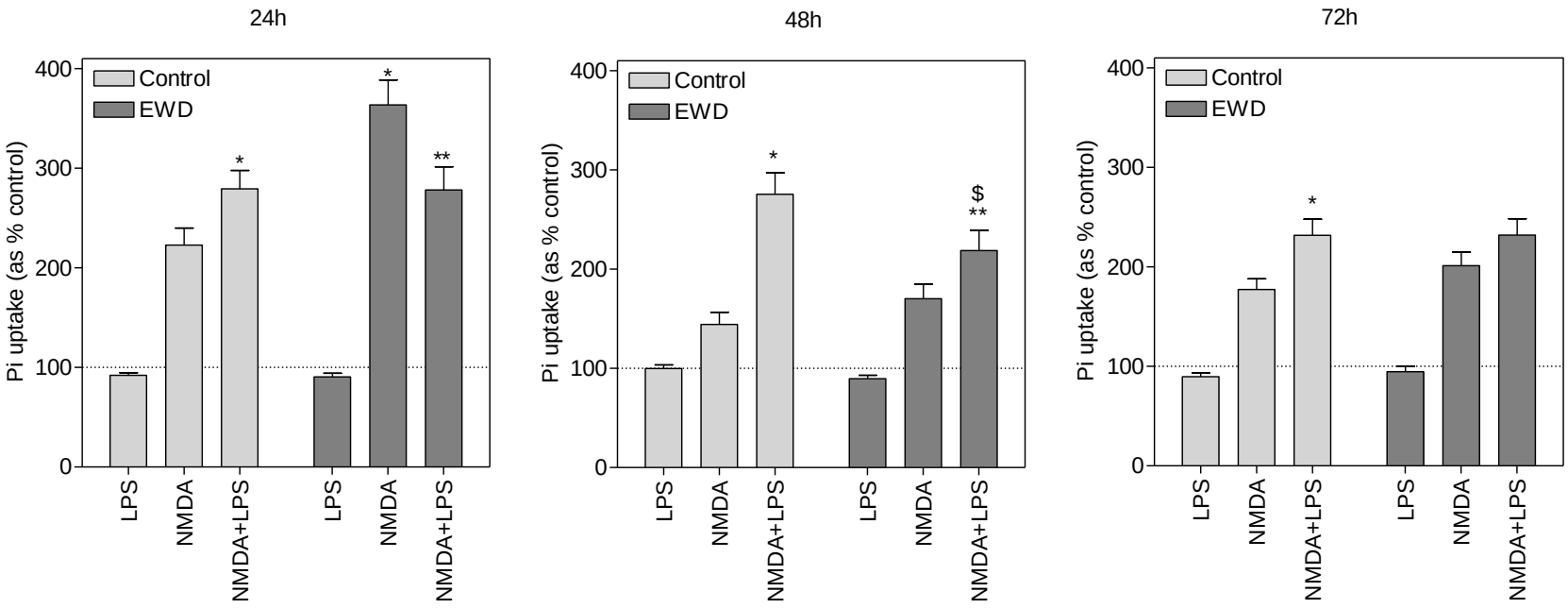

Figure 2.2 Propidium iodide uptake in the CA1 region of the hippocampus following treatment with lipopolysaccharide (LPS), N-methyl-D-aspartate (NMDA) or both.

Slices were treated with LPS, NMDA or a combination of both (NMDA+LPS) for 24h, 48h and 72h under control conditions (light bars) and under ethanol withdrawal (EWD) conditions (dark bars). Propidium iodide is expressed as percent of untreated control (means ± SEM). Dotted line represents untreated control. *p < 0.01 compared to NMDA alone. **p < 0.05 compared to NMDA alone during EWD. $p < 0.01 compared to NMDA+LPS. n = 64-72 slices for each treatment group (untreated, NMDA, LPS, NMDA+LPS), under both control and EWD conditions, at all time points.

2.3.1.3 LPS treatment reduces peak NMDA-induced cellular damage during EWD

Despite potentiation of NMDA-induced cellular damage by LPS under control conditions, the reverse was observed during peak toxicity under EWD conditions (fig. 1). Slices co-exposed to NMDA and LPS during EWD exhibited lower PI uptake (278%) than slices treated with NMDA alone during EWD (363%, post hoc [$p < 0.0001$]). This relationship was however not observed at later time points. At the 48h and 72h time points, slices co-exposed to NMDA and LPS during EWD exhibited higher PI uptake (48h = 218% and 72h = 231%) than slices treated with NMDA alone during EWD (48h = 170%, post hoc [$p < 0.05$] and 72h = 201%, post hoc [$p < 0.05$]). Interestingly, at the 48h time point, slices co-exposed to NMDA and LPS during EWD (218%) exhibited significantly lower PI uptake than slices co-exposed to NMDA and LPS under control conditions (275%, post hoc [$p < 0.01$]).

2.3.2 Effects of NMDA, LPS and EWD on inflammatory mediator release from OHSCs

An overall statistical analysis on TNF-alpha and NO levels measured in culture media was first performed across the treatment groups to assess whether there were effects of sex. This analysis revealed that there was no main effect of sex on TNF-alpha release [$F(1,162) = 3.492$, $p = 0.063$] or NO release [$F(1,358) = 0.200$, $p = 0.655$]. Therefore, further analyses were performed collapsed across this factor.

2.3.2.1 EWD reduces LPS-induced proinflammatory mediator release

LPS treatment induced TNF-alpha release both under control conditions and during EWD (fig. 2.3A). However, in comparison to control conditions, the response was reduced during EWD (main effect of EWD [$F(1,170) = 238.2$, $p < 0.0001$]) at all time points (post hoc at 24h, 48h and 72h [$p < 0.0001$]). Similarly, LPS treatment induced NO

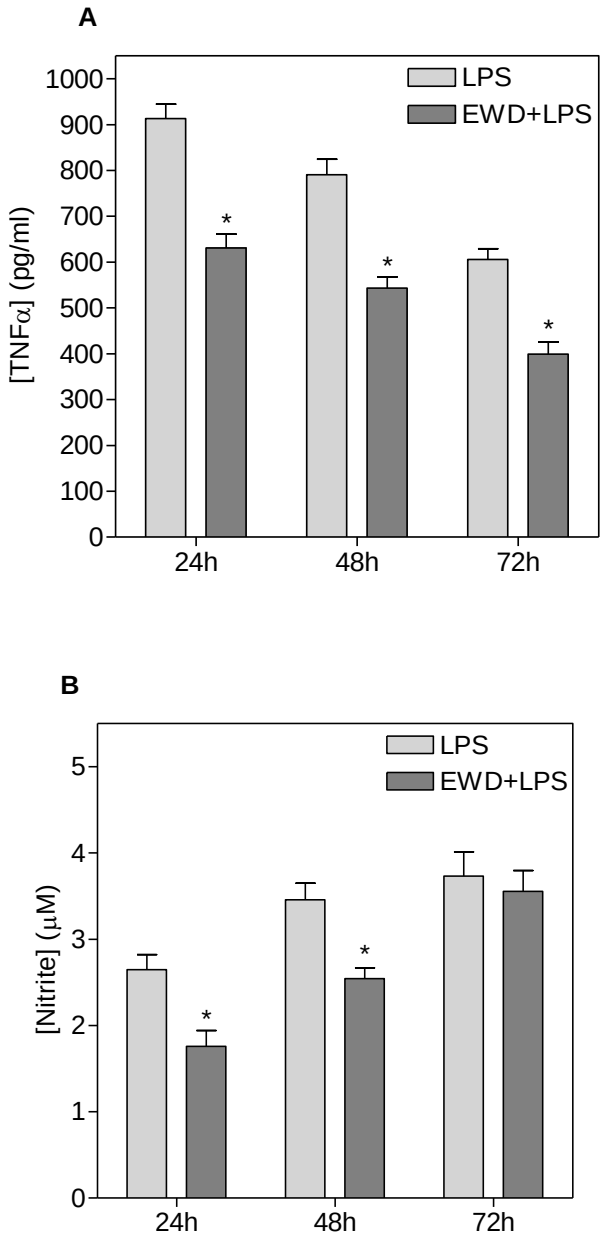

Figure 2.3 Release of proinflammatory cytokines from organotypic hippocampal slice cultures following treatment with lipopolysaccharide (LPS).

Samples were collected after 24h, 48h and 72h of LPS treatment under control conditions (light bars) or under ethanol withdrawal (EWD) conditions (dark bars) and assessed for TNFalpha (A) and NO (B) content. *p < 0.0001 compared to LPS alone; n.d. not detected. n = 16-18 samples for each treatment group (untreated, LPS), under both control and EWD conditions at all time points.

release under control conditions and during EWD (fig. 2.3B - main effect of LPS [$F(1,374) = 1508.1$, $p < 0.0001$]). However, in comparison to control conditions, this effect was also reduced by EWD (EWD x LPS interaction [$F(1,374) = 42.7$, $p < 0.0001$]) at least at earlier time points (post hoc at 24h and 48h [$p < 0.0001$]). At the 72h time point, NO release from EWD slices normalized to control condition levels and there was no significant difference at that time (post hoc at 72h [$p = 0.689$]).

2.3.2.2 NMDA treatment does not induce proinflammatory mediator release and does not interact with the response to LPS

Under control conditions, NMDA alone did not induce TNF-alpha release (TNF-alpha not detected) and had no effect on LPS-induced TNF-alpha release. There were no statistical differences between TNF-alpha levels measured in media from slices co-exposed to NMDA and LPS and slices treated with LPS alone (post hoc at 24h [$p = 0.133$], at 48h [$p = 0.647$] and at 72h [$p = 0.167$]). The same pattern was observed in slices undergoing EWD. There were no statistical differences between TNF-alpha levels measured in media from slices co-exposed to NMDA and LPS and slices treated with LPS alone during EWD (post hoc at 24h [$p = 0.142$], 48h [$p = 0.471$] and 72h [$p = 0.315$]). Similar results were observed for NO release under control conditions (no main effect of NMDA [$F(1,374) = 0.487$, $p = 0.486$]; no NMDA x LPS interaction [$F(1,374) = 0.331$, $p = 0.566$]) and during EWD (no EWD x NMDA interaction [$F(1,374) = 0.196$, $p = 0.658$]; no EWD x NMDA x LPS interaction [$F(1, 374) = 0.844$, $p = 0.359$]).

2.3.3 Effects of LPS and EWD on inflammatory mediator release from BV2 microglia

BV2 microglia exposed to ethanol for 10 days did not exhibit morphological changes compared to control cultures. Additionally, cell viability, as measured by resazurin fluorescence did not differ between ethanol exposed and control cultures during these experiments. LPS treatment induced release of TNF-alpha and NO under

both control and EWD conditions (fig. 2.4). BV2 microglia undergoing EWD exhibited a reduction in LPS-induced release of NO compared to controls (main effect of EWD [$F(1, 36) = 214.1$, $p < 0.0001$]). On the other hand, BV2 microglia undergoing EWD exhibited a potentiated release of TNF-alpha induced by LPS compared to control conditions (main effect of EWD [$F(1, 22) = 19.46$, $p < 0.001$).

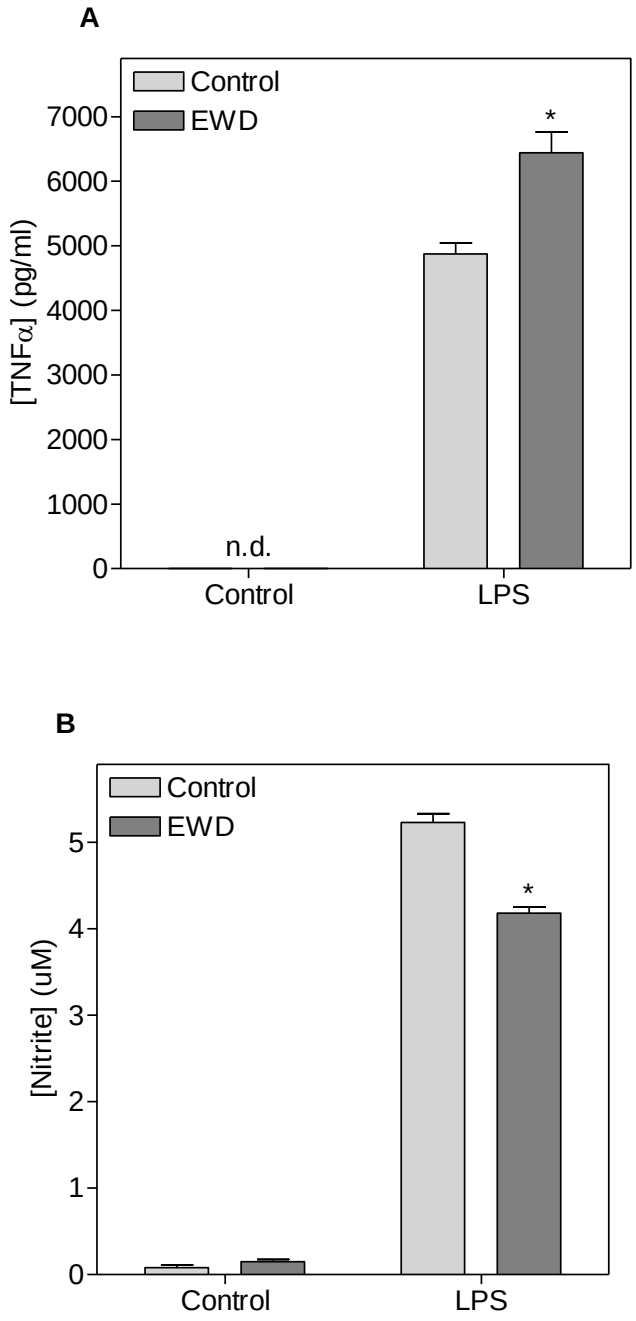

Figure 2.4 Release of proinflammatory cytokines from BV2 microglia following treatment with LPS.

Samples were collected after 24h of LPS treatment under control conditions (light bars) or under ethanol withdrawal (EWD) conditions (dark bars) and assessed for TNF-alpha (A) and NO (B) content. *p < 0.05 compared to LPS alone; n.d. not detected. Treatment groups were run in quadruplicates.

2.4 Discussion

The present studies were undertaken to test the hypothesis that ethanol exposure causes neuroinflammation which in turn potentiates excitotoxicity associated with EWD. Specifically, following ethanol exposure, OHSC were treated with NMDA, LPS, or the combination of both and evaluated for toxicity (PI uptake) and release of proinflammatory mediators (TNF-alpha and NO).

Prior to testing the effects of NMDA and LPS during EWD, we established their combined effects under control conditions. Our original prediction for this set of experiments was that LPS would potentiate NMDA toxicity. Zou and Crews used hippocampal-entorhinal cortical slices (similar to OHSC) to show that TNF-alpha potentiates glutamate toxicity [121] suggesting that neuroinflammation enhances excitotoxicity. We were able to corroborate and support these findings using NMDA as the excitotoxic insult and LPS as the inflammatory stimulus. LPS treatment alone did not produce quantifiable toxicity as measured by PI uptake but OHSC co-exposed to NMDA and LPS exhibited significantly more toxicity than cultures treated with NMDA alone (fig. 1).

Numerous mechanisms have been proposed for the potentiation of excitotoxicity by neuroinflammation [66]. Of particular interest to this study, NO and TNF-alpha released from immune cells have been found to contribute to excitotoxic injury by increasing extra-synaptic glutamate. In co-culture experiments, NO released from astrocytes and microglia has been shown to induce glutamate release from neurons by inhibiting neuronal respiration [75]. In parallel, TNF-alpha has been shown to down-regulate glutamate reuptake by decreasing EAAT2/GLT1 expression on glia [69]. In our study, release of TNF-alpha and NO was induced by LPS and their presence in culture media could explain LPS-enhanced NMDA toxicity. As an alternative explanation,

activated microglia have been shown to release quinolinic acid [67] and glutamate [68], both of which activate NMDARs. Therefore, LPS activation of microglia may have increased the overall concentration of NMDAR agonists in the culture media thereby potentiating NMDA-induced toxicity. The precise mechanisms by which LPS enhances NMDA toxicity in the current study remain uncertain at this stage. This set of experiments supports the fact that neuroimmune signaling induced by LPS is capable of potentiating NMDAR mediated excitotoxicity and this might have important implications for neurotoxicity associated with EWD. For example, if release of NO and TNF-alpha elicited by LPS is enhanced during EWD, this may further potentiate excitotoxicity observed at that time.

Many previous studies [55, 120] using OHSC have shown that NMDA-induced toxicity is enhanced during EWD and we were able to replicate these findings in the current study. NMDA treatment alone produced robust toxicity and this was significantly potentiated during EWD. Since NMDA toxicity is enhanced by LPS under control conditions and NMDA toxicity is also enhanced by EWD, we predicted that NMDA toxicity would be further enhanced by LPS under EWD conditions. Furthermore, this was predicted to be accompanied by a greater release of proinflammatory mediators induced by LPS because ethanol exposure has been shown to sensitize the inflammatory response to LPS [40]. The results do not support these predictions. During peak EWD, co-exposure to LPS reduced NMDA toxicity (fig. 1) and LPS-induced release of proinflammatory mediators was reduced (fig. 2) at that time. Therefore, in OHSC, ethanol exposure resulted in a desensitized response to LPS and the effects of LPS on NMDA toxicity are reversed during EWD.

A potential explanation for the desensitization to LPS following ethanol exposure is by a mechanism similar to endotoxin tolerance. Endotoxin tolerance occurs when

immune tissues or cells are chronically exposed to an inflammatory stimulus resulting in a desensitized response to subsequent challenges with the same stimulus [132]. For example, Antonietta Ajmone-Cat et al. [133] show in an elegant study that when OHSC are treated repeatedly with LPS, the cultures exhibit a reduced inflammatory response to a subsequent challenge. This is presumably the result of persistent activation of the primary molecular target for LPS, toll-like receptor 4 (TLR4). Ethanol has been shown to directly activate TLR4 on isolated astrocytes [36] and microglia [38]. In our study, it is therefore possible that ethanol persistently activated TLR4 resulting in tolerance to a subsequent challenge with LPS during EWD. In support, ethanol pretreatment has been found to produce tolerance to a subsequent LPS challenge in human monocytes and immortalized macrophages [134].

The desensitized inflammatory response to LPS following ethanol exposure is however not sufficient to explain the reversed effects of LPS on NMDA toxicity during EWD. TNF-alpha and NO are still present in culture media and should still be capable of enhancing excitotoxicity. Therefore, other changes to OHSC induced by ethanol must have had to occur for LPS to reduce NMDA toxicity during EWD. Marshall et al recently reported that in rats exposed to a 4-day binge ethanol paradigm, microglia are partially activated and exhibit an anti-inflammatory phenotype [135]. This phenotype has been extensively demonstrated to be neuroprotective against a variety of neurotoxic stimuli [136, 137]. In our study, ethanol exposure may have produced preparations containing anti-inflammatory and neuroprotective microglia which could explain the apparent neuroprotection by LPS against NMDA toxicity during EWD. This interpretation has important implications for the role microglia may play in neurotoxicity associated with EWD. Therefore, we investigated how ethanol exposure affects the response to LPS on microglia specifically.

If the neuroimmune changes induced by ethanol in OHSC are specific to microglia, we should observe similar effects on the response to LPS in BV2 microglia cultures exposed to ethanol. In OHSC, both TNF-alpha and NO release elicited by LPS were reduced following ethanol exposure. Similarly, in BV2 microglia, LPS-induced NO release was *reduced* following ethanol exposure. However, LPS-induced TNF-alpha release was *increased* following ethanol exposure (fig. 3). Accordingly, following ethanol exposure, BV2 microglia exhibit an unusual inflammatory phenotype. Understanding microglial activation and the resulting phenotype is still an ongoing area of research and a plethora of activation states have been identified and are still being characterized [138, 139]. In the current experiments, NO is reduced and TNF-alpha is increased following ethanol exposure, which is not characteristic of traditional classical and/or alternative activations but instead could suggest another activation state such as an immunoregulatory phenotype [140]. However, additional markers need to be measured to fully characterize the activation state of microglia in these experiments. Nevertheless, the discrepancy between results obtained from OHSC and those obtained from BV2 microglia suggests that other cell types are involved in the neuroimmune changes induced by ethanol in the slice cultures. For example, astrocytes are present in OHSC [125] and they have been shown to be activated by ethanol acutely [36]. Prolonged ethanol exposure may have changed their neuroimmune phenotype and contributed to the overall anti-inflammatory phenotype exhibited by the slices. However, additional studies are necessary to fully characterize neuroimmune changes to all cell types in OHSC, including microglia, astrocytes, oligodendrocytes and neurons, following ethanol exposure.

OHSC used in these studies are taken originally from neonatal rats at postnatal day 8 and, although they are not analyzed until 16, 17 or 18 DIV, the effects of ethanol

on these cultures is almost certainly relevant to effects on the developing brain. Indeed, we have previously used these preparations to study the sensitivity of different developmental stages to the effects of excitotoxicity during EWD [141]. Additionally, others [81] have used similar cultures to study the contribution of neuroinflammation on ethanol-induced neurotoxicity specifically during brain development. Their data focused on the role of increased phospholipase A2 activation in neuroinflammation in adolescent cultures, a change that we have previously reported in adult brain [142]. The current studies therefore provide further support that changes to the glutamate and neuroimmune systems may contribute to ethanol related toxicity in the developing brain. Furthermore, they suggest that the therapeutic targets identified in this research may be relevant to ethanol-induced neurotoxicity at different developmental ages.

In summary, in OHSC, LPS enhances NMDA toxicity under control conditions but the reverse is observed during EWD. In addition, ethanol exposure results in a desensitized inflammatory response to LPS. The primary hypothesis is therefore not supported by the data. Furthermore, the specific role of microglia in neuroimmune changes induced by ethanol in this model remains uncertain but data obtained from BV2 microglia suggest that other cell types are important for these changes in OHSC. This has important implications for the treatment of ethanol-induced neurotoxicity because, following ethanol exposure, the neuroimmune system may be endogenously protective against excitotoxicity associated with EWD. Additionally, a comprehensive understanding of the effects of ethanol on all neuroimmune cell types is necessary to decide which cellular targets to engage. These studies do not invalidate neuroinflammation or excitotoxicity as potential targets for the treatment of ethanol induced neurodegeneration but illustrate the necessity to clearly assess the extent,

importance and interaction between these mechanisms when using models of ethanol induced neurotoxicity.

CHAPTER 3. A NICOTINIC RECEPTOR-MEDIATED ANTI-INFLAMMATORY EFFECT OF THE FLAVONOID RHAMNETIN IN BV2 MICROGLIA

Published paper: Lutz J.A., Kulshrestha M., Rogers D.T., Littleton J.M. *A nicotinic receptor-mediated anti-inflammatory effect of the flavonoid rhamnetin in BV2 microglia.* Fitoterapia 2014; 98c: 11-21.

3.1 Introduction

In the last decade, plant natural products have emerged as a valuable source for neuroprotective compounds [143]. Polyphenols (flavonoids, anthocyanins, chalcones, curcuminoids, stilbenoids) in particular have been widely investigated in this area [144] and they generally not only exhibit great antioxidant properties [145] but they also exhibit potent anti-inflammatory properties [146]. Therefore, they have come to light as valuable multifunctional compounds targeting two neurotoxic mechanisms (oxidative stress and neuroinflammation) associated with neurodegeneration.

Neuroinflammation has become known as a central mechanism of neurodegeneration in diseases such as Alzheimer's disease [147], Parkinson's disease [148] or ethanol induced neurotoxicity [34]. It is mediated primarily by microglia, the resident immune cells of the central nervous system (CNS). As a result of acute neuronal damage, microglia become alternatively activated resulting in tissue repair and clean-up [149]. However, when the neuronal insult becomes chronic, as observed in neurodegenerative diseases, microglia become classically activated ultimately leading to additional toxicity [27]. Therefore, the primary aim when targeting neuroinflammation is inhibiting the neurotoxic effects of classical microglial activation. Common, well-known anti-inflammatory drugs, such as ibuprofen, have been suggested for the treatment of neurodegenerative diseases [150]. However, the discovery of novel anti-inflammatory

compounds specific to neuroinflammation has become pivotal in discovering effective treatments for neurodegeneration.

Nicotinic acetylcholine receptors (nAChRs) are emerging as very interesting molecular targets for attenuating neuroinflammation specifically. The homomeric alpha7 nAChR subtype in particular is heavily implicated both in the vagus nerve anti-inflammatory cholinergic pathway [151] and in brain tissues [94]. Wang et al [86] show in an elegant study using an alpha7 nAChR specific antisense oligonucleotide that nicotine inhibits tumor necrosis factor synthesis in activated macrophage cultures through alpha7 nAChRs activation. This finding was then extended to microglia where nicotine and acetylcholine attenuate both microglial activation and LPS-induced tumor necrosis factor alpha (TNF-alpha) release in a dose dependent manner through an alpha7 nAChR dependent pathway [88]. Therefore, an alpha7 nAChR selective agonist would be valuable for targeting neuroinflammation specifically. Moreover, it could have the potential to inhibit excitotoxicity, another primary mechanism of injury observed in neurodegeneration [98].

Although there has been considerable effort toward synthesizing alpha7 nAChR selective ligands in the last decade [108], few of them have made it to market. Interestingly, most of these synthetic compounds are derived from alkaloids originally discovered primarily in plants [109]. The majority of these are toxic or have abuse liability (like nicotine) due partly to their high affinity for alpha4-beta2 nAChRs [110], the other major nAChR subtype in the brain. However, not all plant alkaloids have this selectivity. Thus, methyllycaconitine (MLA) is a relatively selective ligand for the alpha7 nAChR subtype that is isolated from the seeds of Delphinium brownii, a North American wildflower [112]. MLA has been used extensively in research for the study of alpha7 nAChRs but presents little therapeutic use as it is an antagonist [111]. With the

exception of nicotine [152], few of these plant metabolites have been tested against neuroinflammation. However, plants may contain novel, non-toxic, and alpha7 nAChR selective agonists that would have therapeutic value for the treatment of neurodegenerative diseases by attenuating neuroinflammation.

In order to find plants containing metabolites with selective activity at alpha7 nAChRs we developed a differential screening approach and applied this to a large native plant species extract library [113]. These extracts were first screened for the presence of metabolites which bound to nAChRs in rat brain homogenates using a non-subtype selective ligand, [3H]-epibatidine. Of the 1000 extracts tested, about 350 species showed significant displacement of this ligand suggesting that metabolites which interact with nAChRs are common in this sample. Extracts from these 350 species were then compared for their ability to displace an alpha7 selective ligand, [3H]-MLA, or an alpha4-beta2 selective ligand, [3H]-cytisine, from rat brain homogenates. Extracts which displaced the alpha7 selective ligand at lower concentrations than they displaced the alpha4-beta2 selective ligand were considered likely to contain compounds with relative selectivity for alpha7 nAChRs. The great majority of plant extracts showed the reverse selectivity, suggesting that the majority of plant metabolites in this library are, like nicotine, relatively selective for alpha4-beta2 nAChRs. However, 8 species extracts showed relative selectivity for alpha7 nAChRs [113] and these are now under investigation to determine the active metabolites which they contain.

One of the first sources of alpha7 selective binding activity to be investigated was *Solidago nemoralis* which, to our knowledge, has not been reported to contain bioactive alkaloids, the major class of natural products from plants which act on nAChRs. Assay-guided fractionation followed by preparative HPLC and mass spectrometry identified specific methyl-quercetin derivatives as responsible for the displacement of [3H]-MLA

binding. This was surprising for two reasons. First, similar flavonoids are widespread in plants, so it seemed likely that many other plant species should contain alpha7 selective binding activity based on this type of compound. However, our identification of S. nemoralis as “positive” was based not only on binding to alpha7 nAChRs, but also on the relative absence of binding to alpha4-beta2 nAChRs. Since alkaloids which bind to alpha4-beta2 nAChRs are widespread in plants [113] this activity will mask the presence of alpha7 selective compounds in many species. Thus it may be precisely because S. nemoralis contains no bioactive alkaloids that we were able to identify the active flavonoids in this species using our differential screen. The second surprising aspect of this discovery is that flavonoids have never previously been reported to interact directly with nAChRs. However, closely following our observation, electrophysiological studies indicated that quercetin has co-agonist effects on the alpha7 nAChR expressed in Xenopus oocytes [153]. This activity of flavonoids at alpha7 nAChRs is potentially important in several of their known therapeutic effects. For example, flavonoids have been extensively investigated for their anti-inflammatory properties peripherally [154] and in the CNS [155] as well as for their neuroprotective effects in models of neurodegeneration [156]. Many of these effects could be mediated through alpha7 nAChRs but this has never previously been investigated. It will depend on which specific flavonoids have this activity, and the extent to which alpha7 nAChRs are involved in the pathological response. The main aim of this study is therefore to assess the alpha7 nAChR selectivity of a small library of pure flavonoids and whether this activity translates to enhanced anti-inflammatory properties on activated microglia.

3.2 Materials and methods

3.2.1 Chemicals, reagents and kits

Methanol, hexane, chloroform, ethyl acetate, butanol, lipopolysaccharide (LPS) from Escherichia coli serotype 026:B6 (Lot# 021M4072V), (-)-nicotine, methyllycaconitine citrate salt hydrate (MLA) from Delphinium brownii seeds, mecamylamine hydrochloride and 7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt (resazurin) were purchased from Sigma-Aldrich (St Louis, MO, USA). Astragalin, baicalein, catechin, daidzin, daidzein, delphinidin, genistein, isoquercitrin, isorhamnetin, malvidin, petunidin-3-glycoside, quercetagetin, quercetin, rhamnetin, sakuranetin, spiraeoside and tamarixetin (Fig. 1) were purchased from Chromadex (Irvine, CA, USA). [3H]-MLA (~60Ci/mmol), [3H]-cytisine (~16Ci/mmol) and [3H]-epibatidine (~30Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Antibiotics (10000U/mL penicillin and 10000ug/mL streptomycin), 2.5% trypsin (10X), fetal bovine serum (FBS), Dulbeccos' Modified Eagle Medium (DMEM), DMEM:Nutrient mixture F-12 (DMEM/F-12) and Hanks Balanced Salt Solution (HBSS) were purchased from Life Technologies Corporation (Grand Island, NY, USA). Griess reagent system was purchased from Promega Corporation (Madison, WI, USA). TNF-alpha ELISA Ready-SET-Go!® was purchased from eBioscience Inc. (San Diego, CA, USA).

3.2.2 Solidago nemoralis extraction and chemical identification

3.2.2.1 Assay guided fractionation

The aerial parts of *Solidago nemoralis*, including stems and flowers, were dried, ground and suspended in methanol for 24 hours. The methanol extract was decanted and the resulting plant material was re-suspended in fresh methanol for another 24 hours. Methanol extracts were then pooled and dried in a rotary evaporator. The dry

methanol extract was re-suspended in water and subsequently extracted in hexane, chloroform, ethyl acetate and butanol. Resulting fractions were assayed for [3H]-MLA displacement on rat hippocampal membranes (see below).

3.2.2.2 Preparative high performance liquid chromatography (HPLC)

Preparative HPLC was performed with a Waters XBridge preparative c18 column (5µm particles, 19x150mm) and a gradient analysis method (flow rate= 7mL/min) where methanol was gradually increased and water proportionally decreased. Detection was performed with the Waters 2998 photodiode array detector and fractions were collected with the Waters 2767 sample manager. Resulting fractions were assayed for [3H]-MLA displacement on rat hippocampal membranes (see below).

3.2.2.3 Electrospray ionization mass spectrometry (ESI-MS/MS)

Preparative HPLC samples were dried and re-suspended in methanol/MilliQ water and analyzed at the University of Kentucky Mass Spectrometry Facility using ESI-MS/MS in both positive and negative ion ESI modes.

3.2.3 Radioligand binding studies

3.2.3.1 Rat brain membrane preparation

Hippocampal and cortical tissues were removed from adult male Sprague-Dawley rats (225-250g) and homogenized in sucrose buffer (0.32 M sucrose, 1mM EDTA, 0.1mM phenylmethylsulfonyl fluoride, 0.01% w/v sodium azide, pH adjusted to 7.4), centrifuged at 1,000g for 10min at 4°C and the supernatant reserved on ice. The pellet was re-suspended and centrifuged once more at 1,000g for 10min at 4°C. The supernatant was combined with the previously reserved supernatant and centrifuged at 50,000g for 10min at 4°C. The resulting supernatant was discarded and the pellet

washed twice by re-suspension and centrifugation in binding buffer (50mM Tris, 144mM NaCl, 1.5mM KCl, 2mM CaCl2, 1mM MgSO4.7H2O, 20mM HEPES, pH adjusted to 7.4) at 50,000g for 10min at 4°C. Total protein content was measured using the Bicinchoninic Acid Kit (Sigma-Aldrich), adjusted to 3mg/ml protein and frozen at -80°C for future experimentation.

3.2.3.2 General radioligand binding method

Solutions to be tested were mixed with membranes (final protein content = 1mg/ml) and radioligand (2nM [3H]-MLA or 2nM [3H]-cytisine for displacement studies) in individual wells on 96 well plates. Reactions were allowed to reach equilibrium (2h-3h) and the plates were harvested by vacuum filtration onto GF/B filter plate (Perkin Elmer Inc., Waltham, MA, USA) followed rapidly by 3 washes with 50mM Tris-HCl buffer (pH adjusted to 7.4). Filter plates were dried overnight and scintillation counting was performed for 2min per well in a Packard TopCount® NXTTM microplate scintillation and luminescence counter following the addition of 35µL scintillation fluid (Microscint 20, Packard Inc) to each filter well. For each assay plate, non-specific binding was measured in the presence of excess nicotine (300µM final concentration) and specific binding was calculated by subtracting non-specific binding from total binding of radioligand alone. Specific binding in the presence of competitors was converted to percentage of total specific binding of radioligand alone

3.2.3.3 Pure flavonoid competition binding experiments

Flavonoids were solubilized at high concentration (100mM) in 100% DMSO and diluted in binding buffer to obtain stock solutions of 30µM. The stock solutions were subjected to 1:10 serial dilutions to obtain a wide range of flavonoid concentrations.

Using the general radioligand binding method described above, flavonoids were assessed for [3H]-cytisine and [3H]-MLA displacement.

3.2.4 BV2 microglia studies

3.2.4.1 BV2 microglia cell culture

BV2 murine microglia (kindly provided by Dr. Linda Van Eldik) were cultured in DMEM/F-12 supplemented with 10% FBS and antibiotics (penicillin 100U/ml, streptomycin 100ug/ml). Cells were kept at 37°C in a humidified atmosphere of air and 5% CO2. For membrane preparation and RLB studies, cells were propagated in T75 flasks, harvested by cell scraping upon confluency, centrifuged (1200 rpm, swinging bucket) for 4 min, re-suspended in sucrose buffer and frozen for future use. Membranes were then prepared as described above for animal tissue. Membranes were adjusted to a final concentration of 3mg/ml and frozen at -80°C for future experimentation. For LPS elicited inflammatory mediator release, cultured cells were detached upon confluency with 0.25% trypsin (2min at 37°C), seeded in 24 well plates at densities of 5x105 cells/well and allowed to adhere overnight. Cells were then pretreated with test compounds (serum-free media) for 1h and subjected to LPS challenge (10µg/ml in serum-free media) for 24h.

3.2.4.2 Saturation binding with BV2 membranes

Using the general radioligand binding method described above, saturation binding experiments were undertaken as follows: BV2 membranes were mixed in individual wells of a 96 well plate with increasing concentrations of radioligand ([3H]-MLA and [3H]-epibatidine) in the presence or absence of excess nicotine (300µM). Specific binding at each concentration was calculated by subtracting non-specific binding to total binding.

3.2.4.3 Measurement of cell viability, nitric oxide (NO) content and TNF-alpha release

Cell-free culture supernatants were collected, assayed for nitrite content immediately to avoid the effects of freeze-thawing [157] and subsequently frozen for future TNF-alpha content analysis. Each well was then filled with fresh media containing 100µM resazurin and allowed to incubate for 4h. Fluorescence (Excitation = 560nm, emission = 590nm) was then measured using a Wallac 1420 VICTOR plate reader (PerkinElmer, MA, USA) and cell viability was normalized to percentage control (no LPS group). Nitrite levels were measured using the Griess Reagent System (Promega) in accordance with the manufacturer's instructions. Briefly, 50µL of experimental samples were plated in a 96 well plate, 50µL sulphanilamide and 50µL N-1-napthylethylenediamine dihydrochloride were then added sequentially with a 5min incubation interval. Absorbance was measured at 550nm using a Wallac 1420 VICTOR plate reader (PerkinElmer, MA, USA) and the amount of nitrite was calculated from a NaNO2 standard curve. TNF-alpha levels were measured by ELISA using the READY-SET-GO! Mouse TNF-alpha kit (eBioscience, CA, USA) in accordance to the manufacturer's instruction. When necessary, sample dilutions were performed in order to fall within the concentration range of the standard curve.

3.2.5 Statistical analysis and graphical presentation

Statistical analysis and graphical presentation were done in GraphPad Prism 4.03 for Windows (GraphPad Software, CA, USA). Displacement binding data was fitted with non-linear regression using the sigmoidal dose-response with variable slope and with the top and bottom constrained at 100% and 0% respectively. Binding parameters were extrapolated from the curves and flavonoids were ranked in ascending pIC50 values for prioritization into functional studies. For saturation binding experiments, total and specific

binding were fitted with a one site binding hyperbola and non-specific binding was fitted linearly. Binding parameters were extrapolated from specific binding. For dose response curves in functional assays, inflammatory mediator release (NO or TNF-alpha) was normalized by cell viability when statistical differences were found in cell viability between treatment groups by one way ANOVA. Two non-linear regression models (sigmoidal dose-response and sigmoidal dose-response with variable slope) were then compared using an F-test prior to fitting. Response parameters were extrapolated from the best fit. IC50 values were statistically compared using unpaired t-tests on the corresponding pIC50 values.

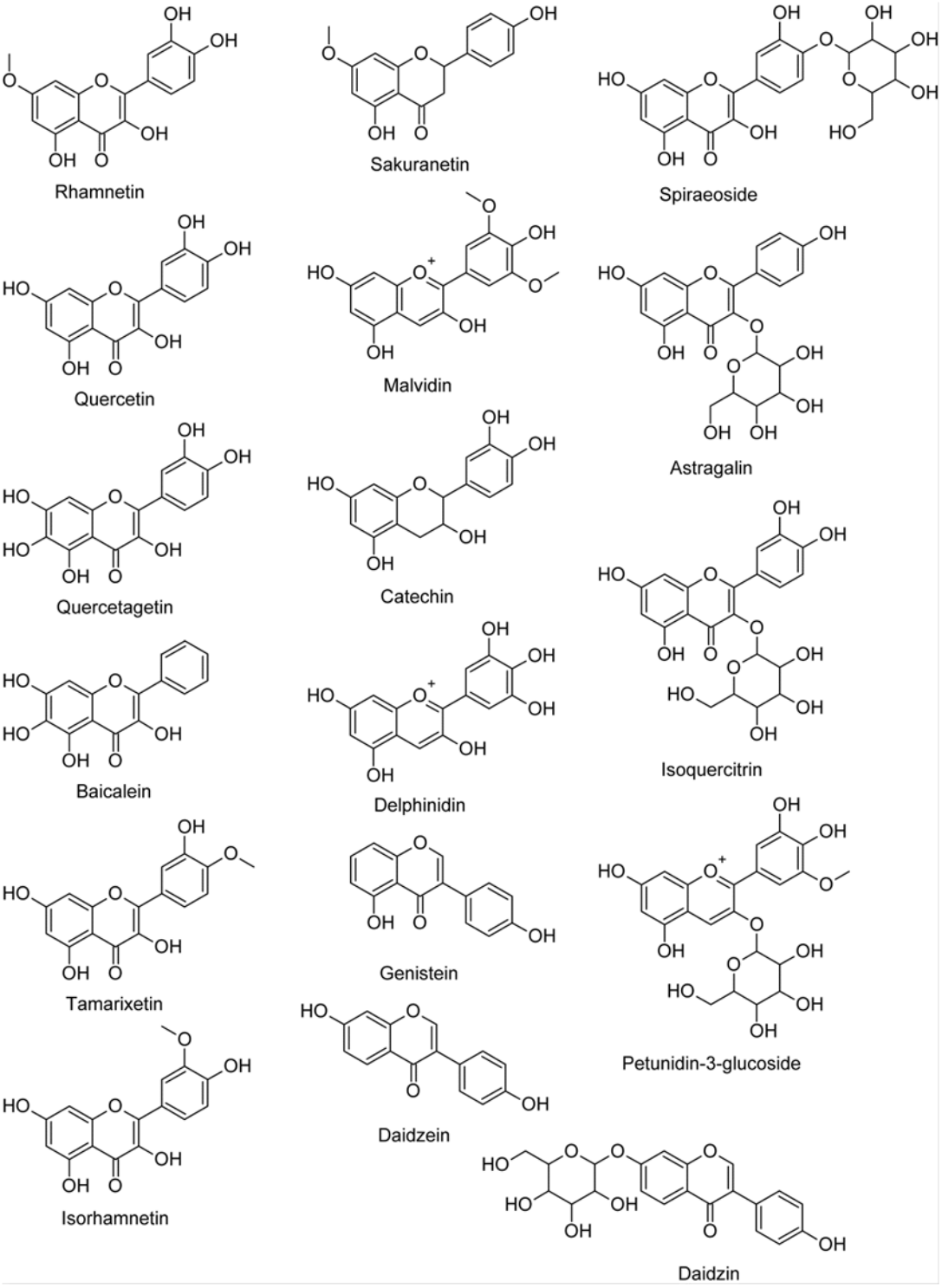

Figure 3.1 Pure flavonoids screened for [3H]-MLA displacement in rat hippocampal membranes

3.3 Results

3.3.1 [3H]-MLA displacing fractions from Solidago nemoralis contain methyl-quercetin derivatives

Solidago nemoralis was previously identified as containing selective binding activity for the alpha7 nAChR relative to beta2-containing nAChRs using our radioligand binding differential smart screen [113]. Therefore, we sought to identify the compound(s) responsible for this activity using [3H]-MLA displacement guided fractionation, pHPLC and ESI-MS/MS. [3H]-MLA displacement on *Solidago nemoralis* extract solvent partitions indicated that the activity was in the ethyl acetate. Further separation of the ethyl acetate fraction using preparative HPLC yielded a single [3H]-MLA displacing fraction. This fraction was sent for ESI-MS/MS analysis which produced 3 major peaks at m/z787, m/z657 and m/z515. Based on a literature search, their fragmentation patterns are indicative of three distinct compounds: a quercetin derivative, a quercetagetin derivative and a dicaffeoylquinic acid (Table 1).

Table 3.1 MS/MS fragments of major MS peaks and compound identification from the literature

M+	Fragments	Compound ID	Citations
787	641, 479, 317,302	Methyl-quercetin triglycoside	[158, 159]
657	495, 333	Methyl-quercetagetin diglycoside	[160, 161]
515	353	Dicaffeoylquinic acid	[162]

A small library of pure dicaffeoylquinic acid derivatives was compiled and tested for [3H]-MLA displacement. None of the compounds tested displaced [3H]-MLA (data not shown) suggesting that the nAChR activity present in the extract is attributable to the methyl-quercetin derivatives identified. We were unable to purify large enough amounts

of the active fractions for definitive identification using NMR. Instead, we compiled a small library of pure flavonoids to be screened for [3H]-cytisine and [3H]-MLA displacement.

3.3.2 Flavonoids differentially displace [3H]-MLA from hippocampal rat membranes

Pure flavonoids were tested for their ability to displace [3H]-cytisine and [3H]-MLA from rat cortical and hippocampal membranes respectively. Most compounds tested did not displace [3H]-cytisine or did so minimally preventing the extrapolation of any kind of binding parameters from fitted displacement curves. However, the majority of compounds tested, but not all, dose dependently inhibited specific [3H]-MLA binding resulting in a wide range of IC50 values (Table 2). Therefore, [3H]-MLA displacement is not a general property of flavonoids and while some structures displace [3H]-MLA (rhamnetin, spiraeoside, quercetagetin), others do not (genistein, daidzein, sakuranetin). The lack of [3H]-cytisine displacement, a ligand selective for beta2-containing nAChRs, in conjunction with the differential displacement of [3H]-MLA observed suggests that these compounds may be more selective for alpha7 nAChRs than for alpha4-beta2 nAChR.

3.3.3 BV2 microglia exhibit functional nicotinic acetylcholine receptors

Microglial cultures purified from rat brain express functional nAChRs and activation of the alpha7 subtype exerts anti-inflammatory effects [163]. However, and as far as the authors know, there is only indirect evidence in the literature for the presence of functional nicotinic receptors in the BV2 immortalized microglial cell line [164, 165]. Therefore, we sought to evaluate the presence of functional nAChRs in BV2 by identifying membrane expressed nAChR with [3H]-MLA saturation binding on BV2 membranes and by assessing the functional anti-inflammatory effects of nicotine on LPS stimulated live cells.

Table 3.2 [3H]-MLA displacement parameters of pure flavonoids from hippocampal rat membranes

Flavonoid	pIC50	IC50 (μM)	Subclass
Rhamnetin	5.953 ± 0.15	1.11	Flavonol
Spiraeoside	5.078 ± 0.13	8.35	Flavonol
Quercetin	4.940 ± 0.05	11.4	Flavonol
Quercetagetin	4.846 ± 0.21	14. 2	Flavonol
Isoquercitrin	4.667 ± 0.22	21.5	Flavonol
Isorhamnetin	4.332 ± 0.17	46.5	Flavonol
Baicalein	4.18 ± 0.09	66.1	Flavonol
Malvidin	4.046 ± 0.11	90.0	Anthocyanidin
Daidzin	3.922 ± 0.20	119.8	Isoflavone
Tamarixetin	3.623 ± 0.23	238.4	Flavonol
Astragalin	3.305 ± 0.78	495.6	Flavonol
Petunidin-3-glucoside	2.464 ± 0.95	3434	Anthocyanidin
Catechin	0.3036 ± 1.7	497100	Flavan-3-ol
Delphinidin	0.8436 ± 4.1	6976000	Anthocyanidin
Genistein	N/A	N/A	Isoflavone
Daidzein	N/A	N/A	Isoflavone
Sakuranetin	N/A	N/A	Flavanone

We obtained evidence for [3H]-MLA specific binding in BV2 microglia membranes. However, we were unable to reach saturation, and thus extrapolate binding parameters, due to a low apparent affinity. The low affinity of [3H]-MLA (nM range) [166] relative to that of [3H]-epibatidine (pM range) [167] for alpha7 nAChRs and the expenses

associated with carrying out [3H]-MLA saturation binding prompted the alternative use of [3H]-epibatidine. BV2 microglia membranes were found to be saturable with [3H]-epibatidine (Fig. 2 – Bmax = 121.2fmol/mg protein; Kd = 6.5nM) indicating that nicotinic binding sites are present on BV2 membranes.

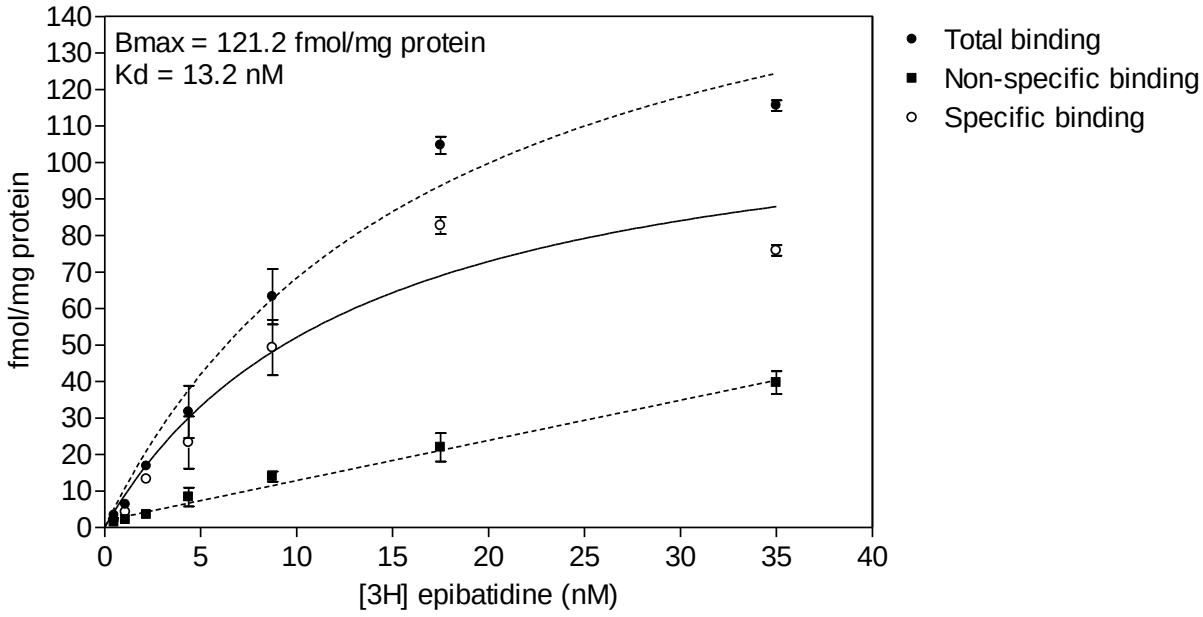

Figure 3.2: [3H]-epibatidine saturation binding on BV2 membranes

BV2 membranes were incubated with increasing concentrations of [3H]-epibatidine in the presence or absence of 300μM nicotine. Specific binding was calculated by subtracting non-specific binding from total binding. Non-specific binding was fit with linear regression while total and specific binding were fit with non-linear one site binding hyperbola.

In cultured BV2 cells, nicotine dose dependently inhibited LPS elicited release of NO with a maximal inhibition of ~20% (Fig. 3). Furthermore, this effect was blocked by 10μM mecamylamine, a non-selective nicotinic antagonist, suggesting that nicotinic

receptors present on BV2 microglia are functional and can be pharmacologically manipulated.

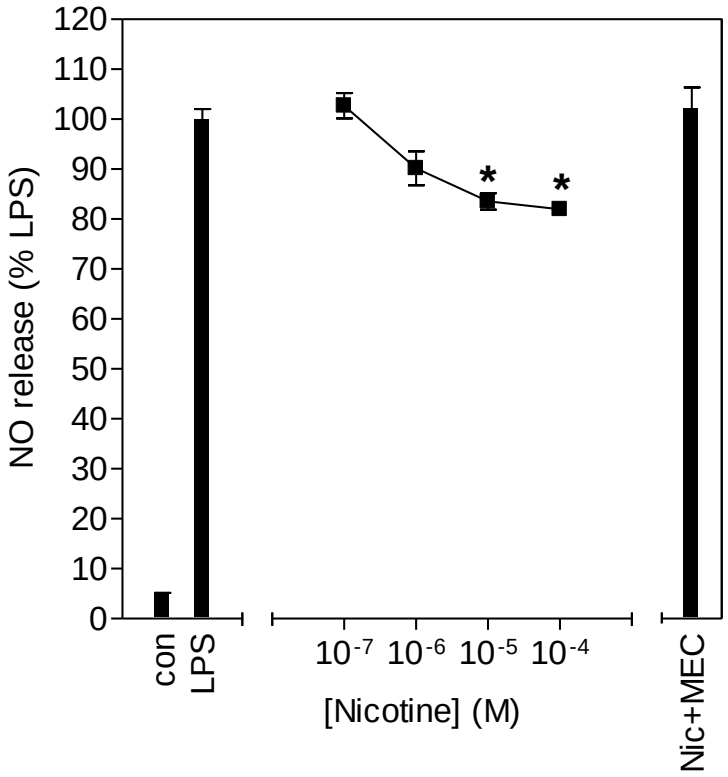

Figure 3.3 Inhibition of NO release from LPS stimulated BV2 microglia by nicotine

BV2 microglia were pretreated for 1h with increasing concentrations of nicotine and subsequently challenged with 10µg/ml LPS for 24h. Culture media was collected and assayed for nitrite content using the Griess reaction. NO release is expressed as percentage of NO release from LPS alone. * p<0.001 compared to LPS alone and Nic+MEC by Tukey's multiple comparison test following a one-way ANOVA.

3.3.4 [3H]-MLA displacement predicts functional anti-inflammatory nicotinic mediated effects

To assess the functional significance of [3H]-MLA displacement studies, rhamnetin, which potently displaces [3H]-MLA with the lowest IC50, and sakuranetin,

which does not displace [3H]-MLA and therefore constitutes a structurally related negative control, were tested for their anti-inflammatory properties on LPS stimulated BV2 microglia. Flavonoids are well known to be anti-inflammatory in this cell line [168, 169]. Therefore, to evaluate the contribution of putative nicotinic activity, their effects were blocked with MLA.

3.3.4.1 Rhamnetin is anti-inflammatory and benefits from a nicotinic mediated mechanism

Rhamnetin dose dependently inhibited NO (IC50 = 26.3µM) and TNF-alpha (IC50 = 28.7µM) release (Fig. 4 & Fig. 5). The addition of MLA increased the IC50s for both inhibition of NO release (IC50 = 33.1µM) and inhibition of TNF-alpha release (IC50 = 105.3µM) although this did not reach statistical significance ($t(6) = 0.94$, $p = 0.37$ for NO; $t(6) = 1.3$, $p = 0.22$ for TNF-alpha). Furthermore, close examination of the concentration response curves without MLA revealed Hill coefficients of 3.0±0.9 and 3.5±4.1 respectively (Table 3). The addition of MLA decreased the Hill coefficients to 1.6±0.4 for inhibition of NO release and the concentration response curve for inhibition of TNF-alpha release best fit the sigmoidal dose response model with no variable slope ($F(1,16) = 2.14$, $p = 0.16$). These data collectively suggest that rhamnetin mediates its anti-inflammatory effects in part through nAChRs.

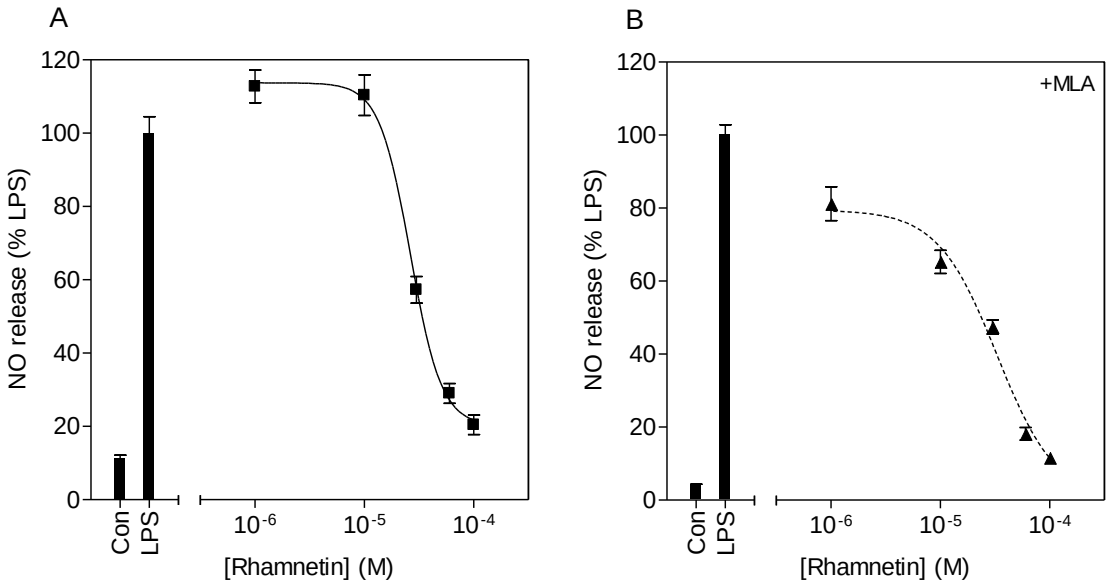

Figure 3.4 Concentration response curves for the inhibition of NO release by rhamnetin

BV2 microglia were pretreated with increasing concentrations of rhamnetin, in the absence (A) or presence (B) of 10μM MLA for 1h and subsequently challenged with 10μg/ml LPS for 24h. Culture media was collected and assayed for nitrite content using the Griess reaction. NO release is expressed as percentage of NO release from LPS alone.

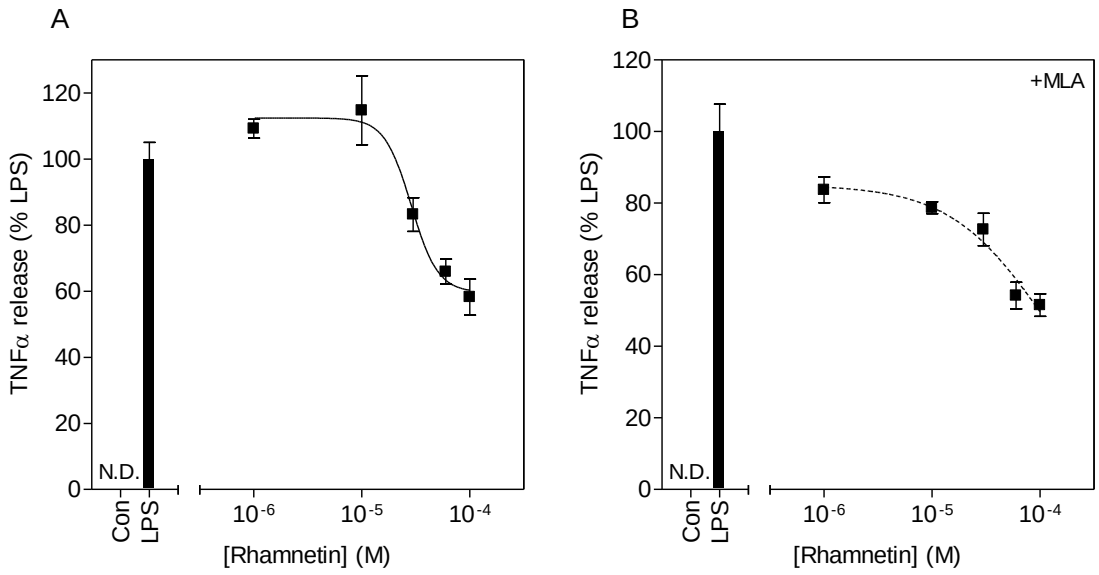

Figure 3.5 Concentration response curves for the inhibition of TNF-alpha release by rhamnetin

BV2 microglia were pretreated with increasing concentrations of rhamnetin, in the absence (A) or presence (B) of 10μM MLA for 1h and subsequently challenged with 10μg/ml LPS for 24h. Culture media was collected and assayed for TNF-alpha content by ELISA. TNF-alpha release is expressed as percentage of TNF-alpha release from LPS alone.

Table 3.3 Concentration response curve parameters for the inhibition of NO release and TNF-alpha release by rhamnetin

*** best fit the sigmoidal dose response curve with no variable slope**

Rhamnetin	IC50 (μM)	pIC50	Hill slope
NO release	26.3	4.579 ± 0.03	-3.0 ± 0.9
+ MLA (10μM)	33.1	4.48 ± 0.10	-1.6 ± 0.4
TNF-alpha release	28.7	4.541 ± 0.06	-3.5 ± 4.1
+ MLA (10μM)	105.3	3.978 ± 0.41	-1.0*

3.3.4.2 The anti-inflammatory activity of sakuranetin does not benefit from a nicotinic mediated mechanism

Sakuranetin dose dependently inhibited NO (IC50 = 85.8μM) and TNF-alpha (IC50 = 158μM) release (Fig. 6 & Fig. 7), albeit with lower relative potency compared to rhamnetin. Close examination of the dose response curves indicated Hill coefficients at unity (Table 4) since both curves fit the sigmoidal dose response model with no variable slope ($F(1,16) = 0.0009$, $p = 0.97$ for NO; $F(1,16) = 3.5$, $p = 0.07$ for TNF-alpha). The addition of MLA had no effect on the anti-inflammatory properties of sakuranetin. However, the addition of a below threshold concentration of nicotine (1μM) decreased IC50s for both NO (IC50 = 16.9μM) and TNF-alpha inhibition (IC50 = 22.3μM). The difference was found to be statistically significant for NO ($t(6) = 2.9$, $p < 0.05$) but not for TNF-alpha inhibition ($t(6) = 1.2$, $p = 0.25$). Furthermore, the Hill coefficients were significantly increased to 2.2±0.3 for inhibition of NO release ($F(1,16) = 15.54$, $p < 0.01$) and 2.7±1.0 for inhibition of TNF-alpha release ($F(1,16) = 6.7$, $p < 0.05$). These data suggest that sakuranetin does not mediate its anti-inflammatory effects through nAChRs

but that a below threshold concentration of nicotine can enhance its pharmacological profile.

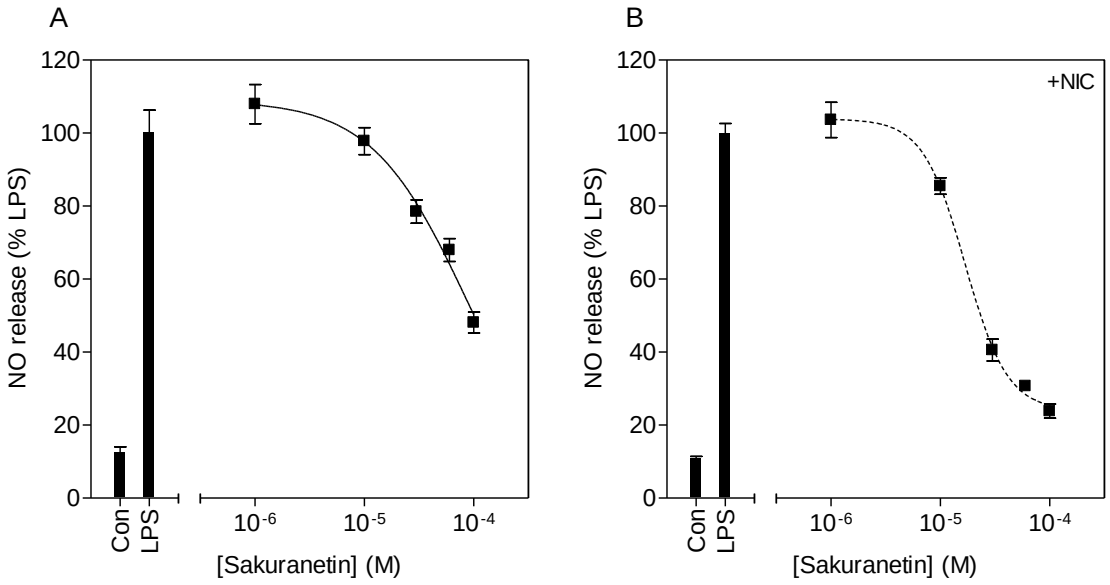

Figure 3.6 Concentration response curves for the inhibition of NO release by sakuranetin

BV2 microglia were pretreated with increasing concentrations of sakuranetin, in the absence (A) or presence (B) of 1μM nicotine for 1h and subsequently challenged with 10μg/ml LPS for 24h. Culture media was collected and assayed for nitrite content using the Griess reaction. NO release is expressed as percentage of NO release from LPS alone.

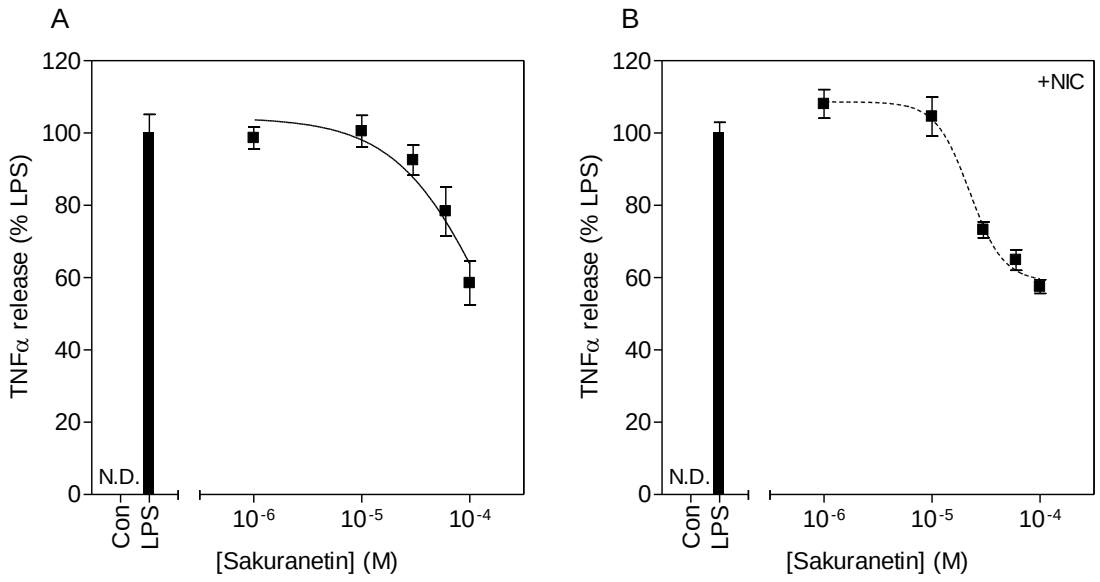

Figure 3.7 Concentration response curves for the inhibition of TNF-alpha release by sakuranetin

BV2 microglia were pretreated with increasing concentrations of sakuranetin, in the absence (A) or presence (B) of 1μM nicotine for 1h and subsequently challenged with 10μg/ml LPS for 24h. Culture media was collected and assayed for TNF-alpha content by ELISA. TNF-alpha release is expressed as percentage of TNF-alpha release from LPS alone.

Table 3.4 Concentration response curve parameters for the inhibition of NO release and TNF-alpha release by sakuranetin

*** best fit the sigmoidal dose response curve with no variable slope**

Sakuranetin	IC50 (μM)	pIC50	Hill slope
NO release	85.8	4.06 ± 0.24	-1.0*
+ Nicotine (1μM)	16.9	4.77 ± 0.04	-2.2 ± 0.3
TNF-alpha release	158.0	3.80 ± 0.67	-1.0*
+ Nicotine (1μM)	22.3	4.65 ± 0.06	-2.7 ± 1.0

3.4 Discussion

Preliminary studies in our laboratory using a radioligand binding differential smart screen on a large plant extract library found that *Solidago nemoralis* extracts produce selective [3H]-MLA displacement relative to [3H]-cytisine displacement suggesting it contains compounds selective for the alpha7 nAChR [113]. Chemical analysis of a semi-pure [3H]-MLA displacing fraction indicated the presence of methyl-quercetin-like flavonoids and a dicaffeoylquinic acid. Lack of [3H]-MLA displacement from a small library of pure dicaffeoylquinic acid derivatives suggested that flavonoids may be responsible for the nAChR activity of the plant extract. Thus, the present studies were conducted to test the hypothesis that specific flavonoid structures selectively bind alpha7 nAChR relative to alpha4-beta2 nAChRs. Therefore, pure flavonoids were evaluated for their ability to displace [3H]-MLA, a relatively selective ligand for the alpha7 nAChR, and [3H]-cytisine, selective for beta2-containing nAChRs. Our original predictions were that some flavonoids would displace [3H]-MLA only, some flavonoids would displace [3H]-cytisine only and some would displace both with varying degrees of potency.

Specific flavonoid structures, such as rhamnetin, specifically displaced [3H]-MLA dose dependently while other specific structures, such as sakuranetin, did not. Therefore, [3H]-MLA displacement is not a general property of flavonoids despite structural similarities. For example, rhamnetin only has 2 additional hydroxyl groups (3-hydroxyl and 3'-hydroxyl) and an unsaturated B ring in comparison to sakuranetin. Interestingly, it appears that flavonols, as a specific subclass, exhibit overall more potent [3H]-MLA displacement in comparison to other subclasses such as anthocyanidins, isoflavones and flavan-3-ols. Therefore, flavonol specific functional groups, such as 4-carbonyl group and 3-hydroxyl group, which are inexistent in other classes, may be contributing to selective [3H]-MLA displacement. Despite relatively low potencies for [3H]-MLA displacement, those for rhamnetin, spiraeoside, and quercetin for example, fall within the same range as concentrations of flavonoids reported in plasma following dietary supplementation [170] and reported anti-inflammatory potencies *in vitro* [171]. Therefore, specific structures displace [3H]-MLA specifically at physiological relevant concentrations. On the other hand, none of the flavonoids tested appeared to displace [3H]-cytisine. Collectively, these results support our hypothesis that certain flavonoid structures specifically bind alpha7 nAChRs relative to alpha4-beta2 nAChRs. A recent report by Lee et al [153] shows that quercetin is capable of enhancing acetylcholine induced inward currents through human alpha7 nAChRs expressed on xenopus oocytes, potentially through interaction with the Ca^{2+} binding site. Therefore, we cannot exclude the possibility that flavonoids may displace [3H]-MLA non-specifically through an allosteric mechanism at the Ca^{2+} binding site. In turn, this could explain the lack of [3H]-cytisine displacement as beta2-containing nAChR are less permeable to Ca^{2+} than alpha7 nAChRs [172]. Nevertheless, [3H]-MLA displacement may have functional significance for the anti-inflammatory properties of these flavonoids which may translate to added value as pharmacotherapies for neuroinflammation specifically. Therefore, we

decided to evaluate and compare rhamnetin and sakuranetin as prototypical [3H]-MLA-displacing and non-[3H]-MLA-displacing flavonoids against LPS elicited inflammatory mediator release from BV2 microglia.

Flavonoids are plant secondary metabolites that are involved in attracting pollinators, deterring herbivorous insects, and allelopathy [173]. They are found in a variety of plant species as well as throughout the human diet in foods such as fruits and vegetables, tea, and cocoa and have been described for their various health benefits [174]. Sakuranetin is the major flavonoid found in rice [175] and it has been primarily studied for its anti-inflammatory [176], anti-diabetic [177], and anti-bacterial [178] properties. Rhamnetin is found in most buckthorns (rhamnus genus) [179] and has been primarily studied for its anti-cancer [180], antioxidant [181] and anti-inflammatory properties [171]. Thus, both rhamnetin and sakuranetin have been found to be anti-inflammatory. Therefore, we hypothesized that rhamnetin, which displaces [3H]-MLA, would benefit from enhanced anti-inflammatory effects, via a nicotinic mediated mechanism, in comparison to sakuranetin, which does not displace [3H]-MLA. The anti-inflammatory properties of both compounds were evaluated by their ability to dose dependently inhibit NO and TNF-alpha release from LPS stimulated BV2 microglia. Additionally, to assess their putative nicotinic activity, they were pharmacologically manipulated with nicotinic agonists and antagonists. We predicted that (1) rhamnetin would be more potent than sakuranetin, (2) rhamnetin would be partially inhibited by nAChR antagonists and (3) sakuranetin would become more potent with the addition of nAChR agonists.

Prior to examining the anti-inflammatory properties of rhamnetin and sakuranetin, BV2 microglia were validated for cell surface presence of functional nAChRs. There is little evidence in the literature for functional nAChRs in this cell line. Mencel et al [165]

report that the alpha7 nAChR subtype can be detected by western blot in BV2 microglia lysates. However, no pharmacological manipulation of the nicotinic receptors in BV2 microglia has been reported to date despite an attempt to block the anti-inflammatory effects of donepezil with MLA and DHBE in these cells, which did not produce an effect [164]. In our hands, BV2 microglia membranes exhibited [3H]-MLA specific binding and were saturable with [3H]-epibatidine. Furthermore, nicotine was found to inhibit NO release from LPS stimulated cells. Therefore, the BV2 microglia cell line appears to have functional nAChRs that can be pharmacologically manipulated to produce anti-inflammatory effects.

Rhamnetin and sakuranetin both dose-dependently inhibited NO and TNF-alpha release with relatively similar potencies but close examination of their concentration-response curves revealed differences in IC50s and Hill coefficients. Rhamnetin exhibited lower IC50s and higher Hill coefficients than sakuranetin. Additionally, pharmacological blockade of rhamnetin with MLA increased IC50s and decreased Hill coefficients. Furthermore, a non-effective concentration of nicotine (1µM) was able to decrease IC50s and increase Hill coefficients for sakuranetin. The Hill coefficient, first described by Archibald Hill in 1910 [182], is a well known parameter in the field of biochemistry where it is extensively used to describe cooperative binding of a ligand or substrate to a receptor or enzyme. However, in the fields of cellular biology, it is used to describe the ultrasensitivity of a system, such as a signal transduction pathway [183]. In our study, the Hill coefficient is simply used to quantify the steepness of the concentration response curves. The difference between the steepness of the slope for rhamnetin and sakuranetin suggests that there may be different anti-inflammatory mechanisms at work. Moreover, the fact that this can be manipulated by nAChR antagonists (for rhamnetin) and agonists (for sakuranetin) suggests that the difference between the two flavonoids

resides in the difference in alpha7 nAChR agonist activity. These data collectively support our hypothesis that [3H]-MLA-displacing flavonoids benefit from a nicotinic mediated anti-inflammatory mechanism.

The alpha7 nAChR selective activity of rhamnetin has important implications not only for enhancement of anti-inflammatory effects but also for its potential as an alpha7 nAChR selective agonist. Indeed, activation of the alpha7 nAChR selectively has the potential to inhibit excitotoxicity mediated through NMDA receptors. Nicotine has been found to be neuroprotective against NMDA induced excitotoxicity through alpha7 nAChR activation both in rat primary hippocampal cultures [98] and hippocampal slice cultures [99]. This activity of nicotine has been proposed as an explanation for the negative association of cigarette smoking with the incidence of AD and PD [95]. Therefore, rhamnetin, as an alpha7 nAChR selective agonist would not only attenuate neuroinflammation but also inhibit excitotoxicity simultaneously. Together with its well-known antioxidant properties, rhamnetin is potentially a multifunctional neuroprotective agent that can simultaneously attenuate neuroinflammation, oxidative stress and excitotoxicity observed in neurodegenerative diseases.

CHAPTER 4. THE DIETARY FLAVONOID RHAMNETIN INHIBITS BOTH INFLAMMATION AND EXCITOTOXICITY DURING ETHANOL WITHDRAWAL IN RAT ORGANOTYPIC HIPPOCAMPAL SLICE CULTURES

Manuscript in preparation

4.1 Introduction

Alcohol-induced neurodegeneration has a complex etiology involving several neurotoxic mechanisms [114], including neuroinflammation [115] and excitotoxicity [59]. These two mechanisms have been studied extensively and are both thought to significantly contribute to ethanol-induced neurotoxicity. This suggests the need to develop therapeutic strategies that can reduce both these pathological mechanisms. One such strategy is by pharmacologically targeting the alpha7 nicotinic acetylcholine receptor (nAChR).

The alpha7 nAChR has recently emerged as a pharmacological target for the treatment of neurodegenerative disorders as it is expressed on both neurons and neuroimmune cells [87, 184] and can be activated to attenuate excitotoxicity and neuroinflammation. For example, nicotine has been found to reduce excitotoxic injury induced by NMDA on hippocampal neurons and this effect was blocked by methyllycaconitine (MLA), an alpha7 nAChR selective antagonist [98]. In parallel, nicotine has been found to attenuate proinflammatory signaling induced by lipopolysaccharide (LPS) on primary microglia cultures, an effect that was significantly blocked by alpha-bungarotoxin, another alpha7 nAChR selective antagonist [163]. In support, DMXB, a relatively alpha7 selective nAChR agonist, has been found to protect neocortical neurons from glutamate toxicity [185] and to inhibit LPS-induced TNF-alpha release from cultured microglia [186]. Taken together, an alpha7 nAChR selective

agonist should act on neurons and microglia simultaneously to reduce excitotoxicity and neuroinflammation.

In order to discover novel alpha7 nAChR selective natural products, we developed a differential pharmacological high throughput screen and applied it to a large Kentucky plant extract library of about 1000 different species [187]. Interestingly, we discovered that *Solidago nemoralis* extracts exhibited selectivity for alpha7 nAChRs relative to alpha4-beta2 nAChRs, the other major nAChR subtype in the brain, and that the compounds responsible for this activity were methyl-quercetin derivatives [188]. Moreover, by screening a pure flavonoid library we discovered that specific flavonoids, such as rhamnetin, were able to selectively displace alpha7 nAChR selective [^{3}H]MLA whereas related structures, such as sakuranetin, do not. Rhamnetin and sakuranetin were compared for their anti-inflammatory properties against LPS induced inflammatory release from immortalized BV2 microglia and rhamnetin was found to inhibit the response in part via alpha7 nAChRs [188]. In sum, rhamnetin is capable of reducing neuroinflammation but also has the potential to reduce excitotoxicity by activating alpha7 nAChRs and therefore constitutes a good therapeutic candidate to reduce ethanol-induced neurotoxicity.

In the current study, rhamnetin is evaluated for its anti-inflammatory and potential neuroprotective properties in a recently developed model of *in vitro* ethanol-induced neurotoxicity which includes neuroinflammatory and excitotoxic component. This model takes advantage of organotypic hippocampal slice cultures (OHSC) that contain both neurons and neuroimmune cells [125-127] and on which drugs can be directly applied to brain tissue without pharmacokinetic confounds. Previous studies from our laboratory have shown that OHSC express alpha7 nAChR, as measured by [^{125}I]alpha-bungarotoxin autoradiography [99], and that nicotine exposure reduces NMDA receptor

mediated excitotoxicity under control conditions [189] and during ethanol withdrawal (EWD) [100]. These studies focused on excitotoxicity and we therefore added a neuroinflammatory component by applying exogenous LPS in conjunction with NMDA during EWD. As such, this model is well suited to evaluate the potential anti-inflammatory and neuroprotective properties of natural products such as rhamnetin. The anti-inflammatory effects of rhamnetin can be assessed on LPS-induced inflammatory mediator release and the neuroprotective effects of rhamnetin can be assessed on NMDA-induced toxicity, both under control conditions and during EWD. In addition, the overall effects of rhamnetin can be evaluated on both outcome measures simultaneously when OHSC are exposed to both LPS and NMDA.

In the original studies, we found that while LPS enhanced NMDA toxicity under control conditions, the reverse was observed during EWD. Additionally, prior ethanol exposure reduced subsequent inflammatory response to LPS. These data suggest that changes to neuroimmune processes induced by ethanol exposure may be protective against excitotoxicity during EWD. Additional experiments on BV2 microglia exposed to the same ethanol regimen suggested that ethanol exposure induces a non-classical microglial phenotype that may be neuroprotective. In support, Marshall et al report that in rats exposed to a 4-day binge ethanol paradigm, microglia are partially activated and take on an anti-inflammatory phenotype [135]. Exposure to flavonoids and alpha7 nAChR agonists has been shown to induce similar changes in microglia. For example, luteolin, another plant derived flavonoid with a very similar structure to rhamnetin, has been shown to induce global transcriptome changes in microglia indicative of an anti-inflammatory and neuroprotective phenotype [190]. Similarly, AR-R 17779, an alpha7 nAChR selective agonist, polarizes primary microglial cultures towards the reparative Mox phenotype [191]. Therefore, it is unclear how rhamnetin exposure will affect

neuroimmune changes induced by ethanol and how those changes in turn affect excitotoxicity during EWD.

Based on the anti-inflammatory effects of rhamnetin on BV2 microglia [188], this compound is predicted to reduce LPS-induced inflammatory mediator release under control conditions. However, whether rhamnetin (1) retains anti-inflammatory properties during EWD, (2) inhibits NMDA toxicity under control conditions, or (3) prevents enhanced NMDA toxicity during EWD remains to be established. The hypothesis is that rhamnetin will reduce LPS-induced inflammatory mediator release and NMDA-induced toxicity under control conditions and during EWD in hippocampal slice cultures. These studies are designed to test this hypothesis in order to establish whether rhamnetin and similar dietary flavonoids have potential value in the prevention and/or treatment of alcohol-induced neurodegeneration.

4.2 Materials & methods

4.2.1 Organotypic hippocampal slice culture (OHSC) preparation

OHSC were prepared essentially as described by Stoppini et al. [129]. Briefly, hippocampi were aseptically removed from 8-day-old Sprague-Dawley male and female rat pups and sliced at a transverse thickness of 200 µm using a McIlwain tissue chopper (Campden Instruments Ltd., Lafayette, ID). Slices were transferred to sterile culture inserts (4 slices per insert) and placed in 6-well-plates containing culture medium (Minimum Essential Medium (MEM; Life Technologies Corporation, Grand Island, NY), 200mM glutamine (Invitrogen, Carlsbad, CA), 25mM HEPES (ATCC, Manassas, VA), 50uM penicillin/streptomycin (ATCC, Manassas, VA), 36mM glucose, 25% (v/v) Hank's buffered salt solution (HBSS; Gibco BRL, Gaithersburg, MD), 25% heat-inactivated horse serum (HIHS; Sigma, St. Louis, MO)). Cultures were maintained at 37°C in an atmosphere of 5% CO2/95% air in 95% humidity for 5 days to allow slices to adhere to

the insert membrane. The care of animals was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996), as well as the University of Kentucky's Institutional Animal Care and Use Committee.

4.2.2 Ethanol exposure, rhamnetin exposure and ethanol withdrawal (EWD)

At 6 days *in vitro*, slices were placed in culture media with or without the addition of 100mM ethanol, incubated for 10 days and then subjected to EWD for 24h. During exposure, the plates were placed in topless polypropylene containers containing 50mL of 100mM ethanol or dH2O accordingly, and placed inside sealable plastic bags filled with 5% CO2, 21% oxygen, and a balance of nitrogen. The ethanol solution is used as an evaporating source of ethanol to counter the evaporation of ethanol from the culture wells resulting in an average ethanol concentration of 65mM during the 5 days between media changes. At 11 days *in vitro*, slices were sub-cultured into their respective culture media (control and ethanol), further subdivided and exposed to control media or media supplemented with rhamnetin (25uM/100uM) for the last 5 days of ethanol exposure. At the onset of EWD, slices were separated into treatment groups with or without rhamnetin and challenged accordingly: control media, 5uM NMDA (Sigma Aldrich Co. LCC., St. Louis, MO), 10ug/mL lipopolysaccharide from *Escherichia coli* 026:B6 (LPS, Lot #: 021M4072V; Sigma Aldrich Co. LCC., St. Louis, MO) or both NMDA and LPS combined.

4.2.3 Assessment of toxicity by propidium iodide uptake

Propidium iodide (PI - Sigma Aldrich Co. LCC., St. Louis, MO) is a membrane impermeable, DNA intercalating fluorescent molecule that is commonly used in OSHC as a semi-quantitative stain for cellular toxicity and has been significantly correlated to other reliable markers of cell death [130]. It has been extensively used to screen neuroprotective compounds in OHSC [119] and we previously used it to evaluate the

combined effects of NMDA and LPS during EWD [in preparation]. Therefore, PI was chosen to evaluate the neuroprotective properties of rhamnetin in this study. During EWD, slices were treated in culture media containing 3.74uM PI. Slice images were captured using SPOT Advanced software (Version 4.0.9; W. Nuhsbaum Inc., McHenry, IL) connected to an inverted Leica DMIRB microscope (W. Nuhsbaum Inc.) fitted for fluorescence detection (mercury-arc lamp) and connected to a computer via a SPOT 7.2 color mosaic camera (W. Nuhsbaum Inc). PI uptake in the CA1, CA3, and DG cell layers was measured using ImageJ software (Version 1.46; National Institute of Health, Bethesda, MD). Background signal was subtracted from intensities obtained for each cell layer resulting in specific intensities which were used for statistical analysis. These values were then converted to % control (no EWD, no NMDA, and no LPS) within each preparation for graphical representation.

4.2.4 Assessment of inflammatory mediator release

Once slices were imaged, inserts were discarded and the resulting media was collected for assessment of inflammatory mediator release. Nitric oxide (NO) release was assessed by the Griess Reagent System (Promega Corporation, Madison, WI) according to the manufacturer's instructions. Briefly, samples were mixed sequentially with sulfanilamide and N-1-napthylethylenediamine dihydrochloride and incubated for 5min. Absorbance was measured at 550nm using a Wallac 1420 VICTOR plate reader (PerkinElmer, MA, USA). All samples were assayed in duplicate and nitrite content was estimated using a reference $NaNO_2$ standard curve performed with each assay. TNFalpha content was assessed by enzyme linked immunosorbent assay kit (ELISA; Ready-Set-Go!® ELISA, eBioscience Inc., San Diego, CA) according to the manufacturer's instructions. Briefly, samples were pipetted on 96-well plates coated with rat anti-TNFalpha antibodies and detected using the sandwich method (anti-TNFalpha

primary antibody, avidin-HRP linked secondary antibody and tetramethylbenzidine substrate). All samples were assayed in duplicate and TNFalpha content was estimated from a reference TNFalpha standard curve performed with each assay.

4.2.5 Statistical analysis

Data were analyzed using IBM Statistical Package for the Social Sciences (SPSS) Version 21 (IBM Corporation, Armonk, NY) and graphed using Prism (Graphpad Software Inc., La Jolla, CA). TNFalpha and NO release were analyzed by multi-factorial analysis of variance (ANOVA) with EWD, NMDA, LPS and rhamnetin as fixed factors. Data were obtained from different preparations so preparation was used as a covariate to control for differences across litters/culture preparations. Post hoc analyses were conducted using Fisher's least significant difference (LSD) test with a level of significance set at $p<0.05$. PI uptake was measured in three different regions (DG, CA3 and CA1). Thus, PI uptake was analyzed by multi-factorial, repeated measures ANOVA with region as within-subjects variables, EWD, NMDA, LPS and rhamnetin as between-subjects factors, and preparation as a covariate. Post hoc analyses were conducted using Fisher's least significant difference (LSD) test with a level of significance set at $p<0.05$.

4.3 Results

Overall multi-factorial ANOVAs on NO release and TNFalpha release revealed that the highest order interactions included all factors except NMDA (for NO release: EWD x LPS [$F(1,502) = 103.6$, $p < 0.001$]; EWD x rhamnetin [$F(2,502) = 7.6$, $p < 0.01$]; LPS x rhamnetin [$F(2,502) = 28.1$, $p < 0.001$]; for TNFalpha release: EWD x rhamnetin [$F(2,237) = 5.6$, $p < 0.01$]). Therefore, differences in inflammatory mediator release between different treatment groups, excluding NMDA groups, were compared post hoc where indicated. The repeated-measures multi-factorial ANOVA on PI uptake revealed a

main effect of region ([F(1.2,2545.9) = 102.01, p < 0.001] corrected using Greenhouse-Geisser). This effect was mainly driven by differences in the CA1 region of the hippocampus where toxicity was most significant compared to DG and CA3. Therefore, assessment of cellular damage was focused on the CA1. A multi-factorial ANOVA on PI uptake in the CA1 revealed that the highest order interaction included all factors (EWD x NMDA x LPS x rhamnetin [F(2,2101) = 6.7, p < 0.01]). Therefore, differences in PI uptake between the different treatment groups were compared post hoc where indicated.

4.3.1 Rhamnetin inhibits LPS-induced inflammatory mediator release but has no effect on NMDA-induced toxicity under control conditions

The anti-inflammatory and neuroprotective properties of rhamnetin were first evaluated under control conditions. Both 25uM and 100uM rhamnetin equally inhibited inflammatory mediator release induced by LPS (Fig. 4.1) but it did not afford neuroprotection against NMDA toxicity under these conditions (Fig. 4.2). TNFalpha and NO levels measured in media from cultures treated with LPS and rhamnetin were significantly lower than those measured in media from cultures treated with LPS alone (post hoc p < 0.0001). As in the original studies, under control conditions, LPS treatment potentiated NMDA-induced PI uptake (post hoc p < 0.05). 25uM or 100uM rhamnetin had no effect on NMDA-induced toxicity. Moreover, despite its anti-inflammatory effects, rhamnetin had no effect on toxicity induced by NMDA and LPS in combination.

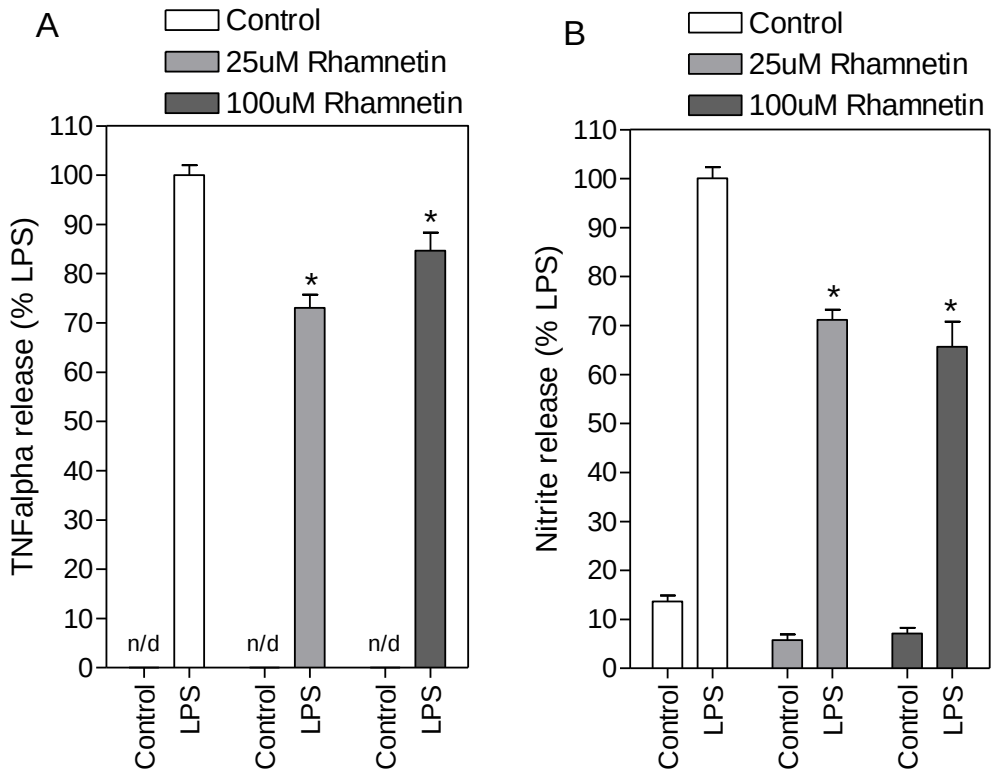

Figure 4.1 Rhamnetin is anti-inflammatory under control conditions

Slices were treated with lipopolysaccharide (LPS) in the absence (white bars) or presence (grey bars) of rhamnetin (25uM and 100uM), culture media was collected after 24h and assayed for TNFalpha (A) and NO (B) content. * p<0.001 compared to LPS alone, n/d not detected. Data are expressed as percent release induced by LPS alone (means ± SEM). n > 12 samples for each treatment group.

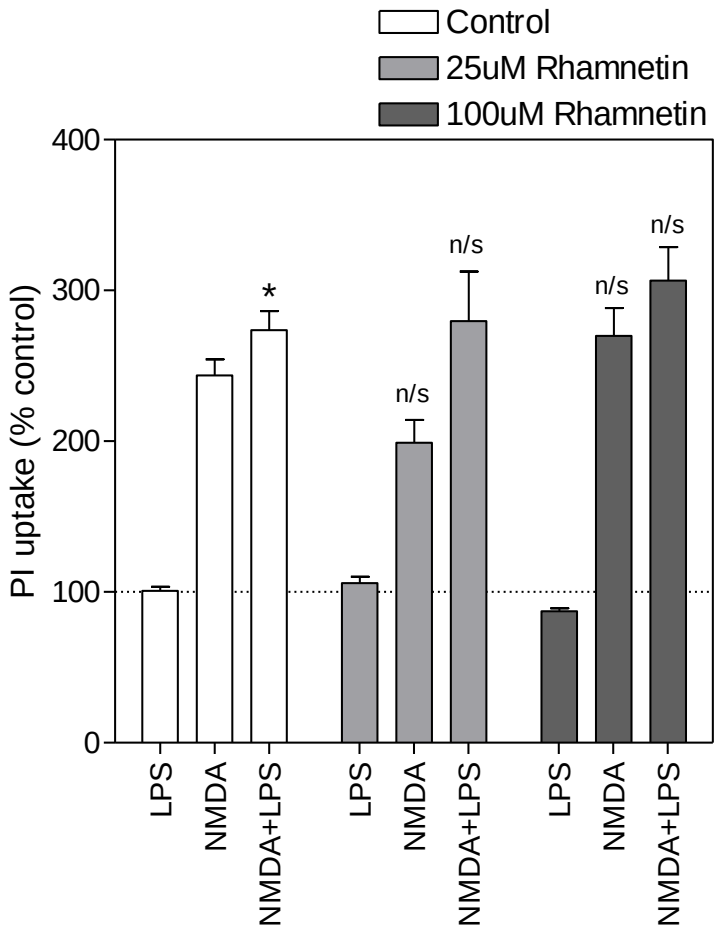

Figure 4.2 Rhamnetin is not neuroprotective under control conditions

Slices were treated with lipopolysaccharide (LPS) and/or N-methyl-D-aspartate (NMDA) in the absence (white bars) or presence (grey bars) of rhamnetin (25uM and 100uM) and PI uptake was measured after 24h. * p<0.05 compared to NMDA alone, n/s no significant difference with equivalent treatment group (LPS, NMDA, NMDA+LPS) in the absence of rhamnetin. Data are expressed as percent of untreated control (means ± SEM). Dotted line represents untreated control. n > 27 for each treatment group.

4.3.2 Effects of EWD on LPS-induced inflammatory mediator release and NMDA-induced toxicity

As in the original studies, EWD reduced the release of inflammatory mediators induced by LPS in comparison to control (Fig. 4.3). TNFalpha and NO levels measured in media from EWD cultures treated with LPS were significantly lower than LPS control levels (post hoc $p < 0.001$). In addition, EWD enhanced NMDA-induced toxicity and the effects of LPS on NMDA-induced toxicity were reversed under these conditions (Fig. 4.4). EWD cultures treated with NMDA exhibited significantly more PI uptake than NMDA controls (post hoc $p < 0.001$) and EWD cultures treated with a combination of NMDA and LPS exhibited significantly less PI uptake than EWD cultures treated with NMDA alone (post hoc $p < 0.01$).

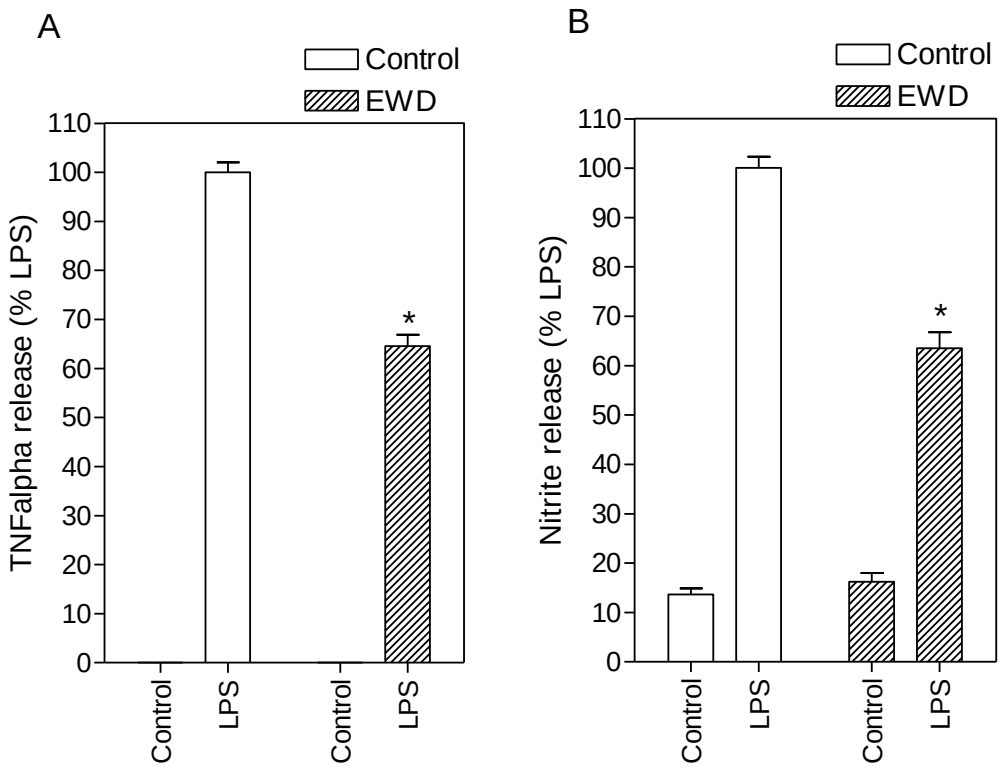

Figure 4.3 Ethanol withdrawal (EWD) reduces lipopolysaccharide (LPS) induced proinflammatory release from organotypic hippocampal cultures

Slices were treated with LPS under control conditions (empty bars) and during EWD (dashed bars), culture media was collected after 24h and assayed for TNFalpha (A) and NO (B) content. *p<0.001 compared to LPS alone. Data are expressed as percent release induced by LPS alone (means ± SEM). n > 27 for each treatment group.

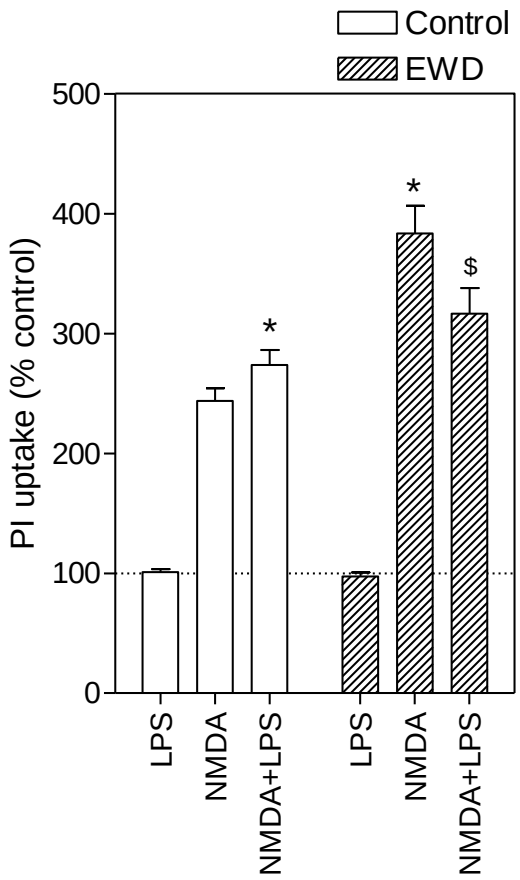

Figure 4.4 Effects of ethanol withdrawal (EWD) on toxicity induced by N-methyl-D-aspartate (NMDA) and/or lipopolysaccharide (LPS) in organotypic hippocampal cultures

Slices were treated with LPS and/or NMDA under control conditions (empty bars) or during EWD (dashed bars) and PI uptake was measured after 24h. *$p<0.05$ compared to NMDA alone; $^{\$}p<0.01$ compared to NMDA+EWD. Data are expressed as percent of untreated control (means ± SEM). Dotted line represents untreated control. $n > 117$ for each treatment group.

4.3.3 Rhamnetin inhibits LPS-induced inflammatory mediator release and NMDA-induced toxicity during EWD

The anti-inflammatory and neuroprotective properties of rhamnetin were evaluated under EWD conditions. Despite the fact that the response to LPS is reduced during EWD, both 25uM and 100uM rhamnetin further inhibited inflammatory mediator release during EWD (Fig. 4.5). TNFalpha and NO levels measured in media from EWD cultures treated with LPS and rhamnetin were significantly lower than those measured in media from cultures treated with LPS alone (post hoc $p < 0.001$). Moreover, both 25uM and 100uM reduced NMDA-induced toxicity during EWD (Fig. 4.6). PI uptake in slices treated with NMDA and rhamnetin was significantly lower than in slices treated with NMDA alone under these conditions (post hoc $p < 0.001$). However, rhamnetin had no effect on toxicity induced by NMDA and LPS in combination during EWD.

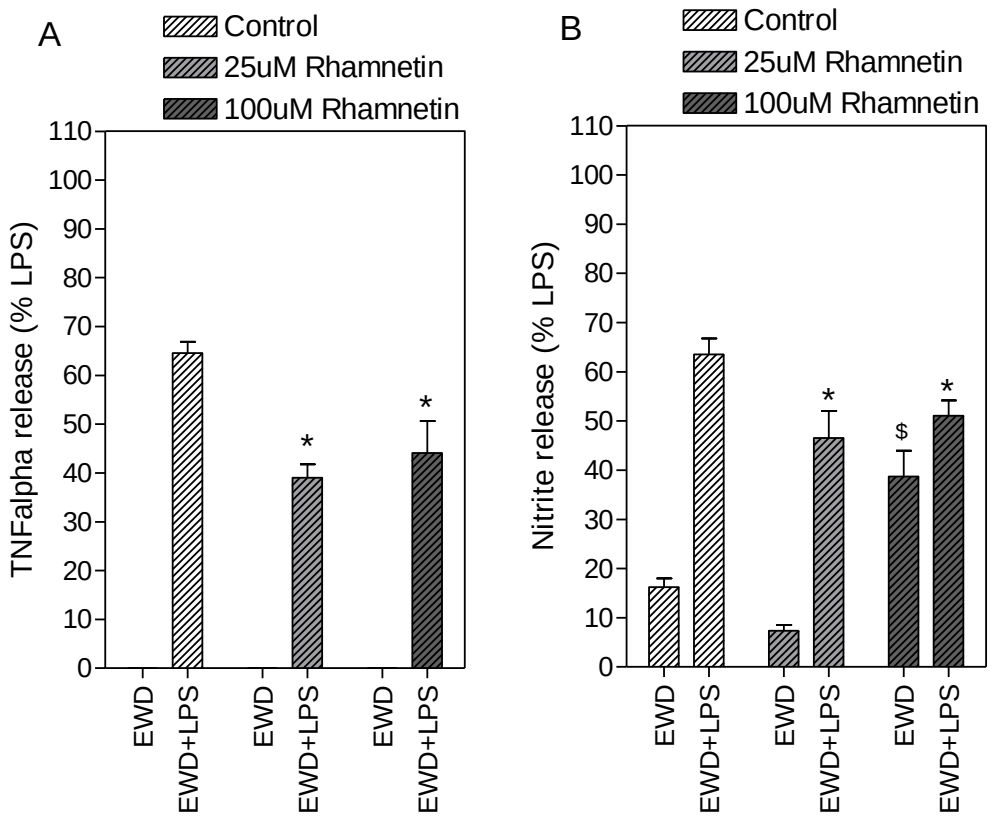

Figure 4.5 Rhamnetin is anti-inflammatory during ethanol withdrawal (EWD)

Slices were treated with lipopolysaccharide (LPS) in the absence (white dashed bars) or presence (grey dashed bars) of rhamnetin (25uM and 100uM), culture media was collected after 24h and assayed for TNFalpha (A) and NO (B) content. *p<0.01 compared to LPS+EWD; $p<0.001 compared to EWD alone. Data are expressed as percent release induced by LPS alone under control conditions (means ± SEM). n > 11 samples for each treatment group.

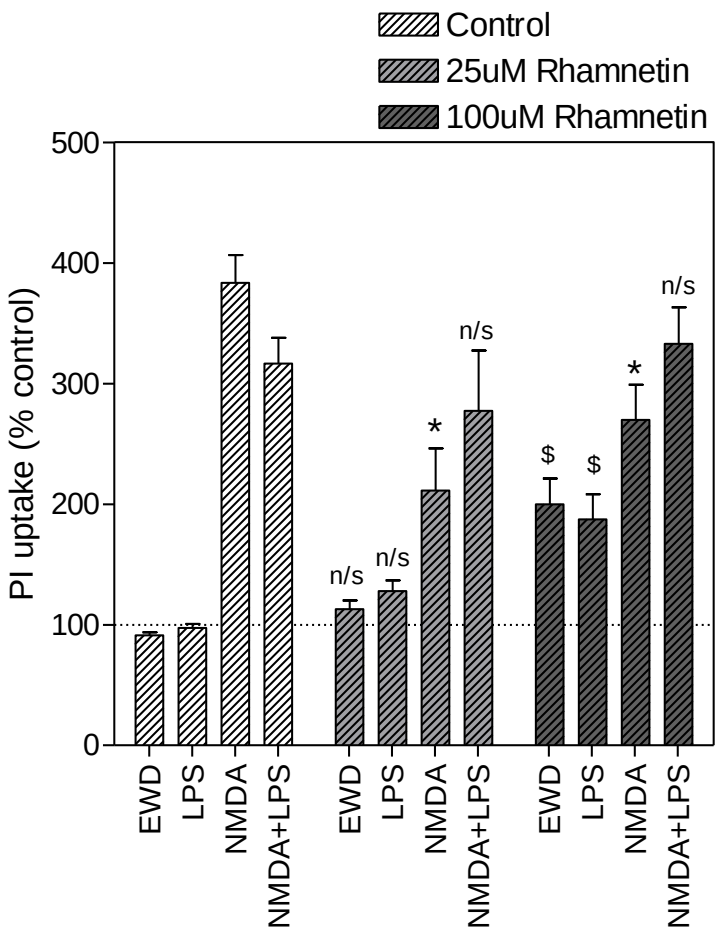

Figure 4.6 Rhamnetin is neuroprotective against N-methyl-D-aspartate (NMDA) toxicity during ethanol withdrawal (EWD)

Slices were treated with lipopolysaccharide (LPS) and/or NMDA in the absence (white dashed bars) or presence (grey dashed bars) of rhamnetin (25uM and 100uM) and PI uptake was measured after 24h. *p<0.001 compared to NMDA+EWD; $p<0.001 compared to EWD alone, n/s no significant difference with equivalent treatment group in the absence of rhamnetin. Data are expressed as percent of untreated control (means ± SEM). Dotted line represents untreated control. n > 45 for each treatment group.

4.3.4 100uM rhamnetin spontaneously induces NO release and toxicity during EWD

Interestingly, 100uM rhamnetin interacted with ethanol exposure and/or EWD to spontaneously induce NO release and toxicity (Fig. 4.7). NO levels measured in media from EWD cultures treated with 100uM rhamnetin was significantly higher than NO measured in media from EWD control cultures (post hoc $p < 0.001$). Similarly, PI uptake was significantly higher in slices treated with 100uM rhamnetin compared to EWD control slices (post hoc $p < 0.001$). On the other hand, 25uM rhamnetin did not interact with EWD and levels of NO release in media or PI uptake in EWD cultures treated with this concentration of rhamnetin did not differ from EWD controls.

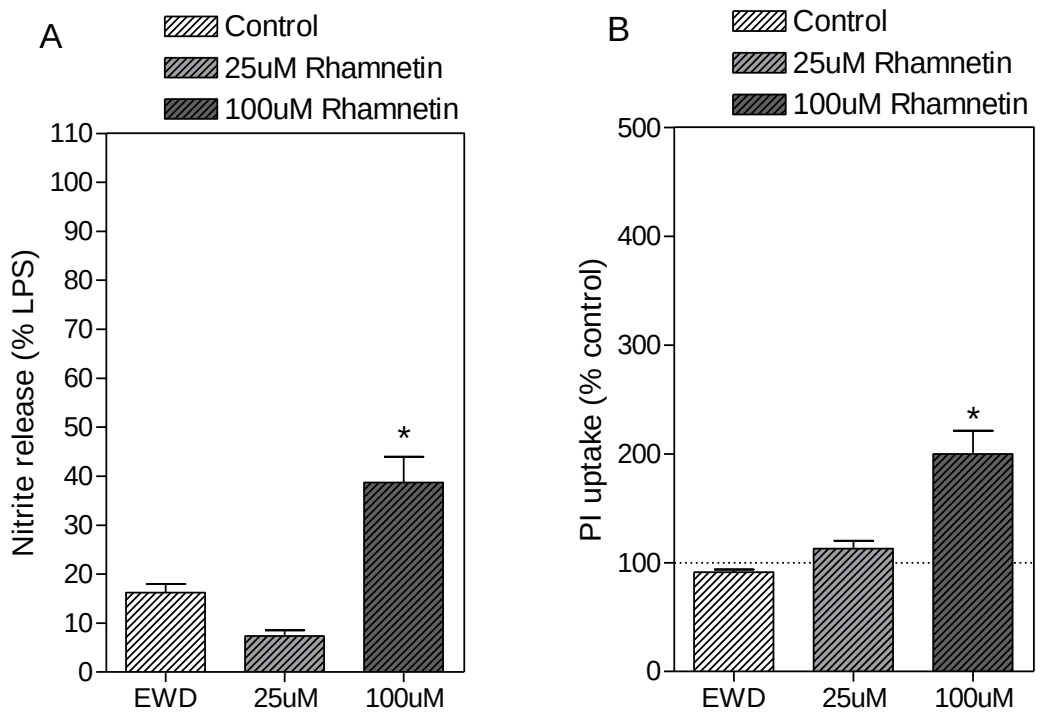

Figure 4.7 High concentration of rhamnetin induces spontaneous NO release and toxicity during ethanol withdrawal (EWD)

Slices were subjected to EWD in the absence (white dashed bars) or presence (grey dashed bars) of rhamnetin (25uM and 100uM). (A) Culture media was collected after 24h and assayed for NO content. *p<0.001 compared to EWD alone. Data are expressed as percent release induced by LPS alone (means ± SEM). n > 12 for each treatment group. (B) PI uptake was measured after 24h. *p<0.001 compared to EWD alone. Data are expressed as percent of untreated control (means ± SEM). Dotted line represents untreated control. n > 47 for each treatment group.

4.4 Discussion

The present studies were undertaken to evaluate the anti-inflammatory and potentially neuroprotective properties of rhamnetin, a dietary flavonoid, in an *in vitro* model of ethanol-induced neurotoxicity that includes both neuroinflammatory and excitotoxic components. Specifically, OHSC were pretreated with rhamnetin for 5 days before initiating EWD, at which point cultures were challenged with NMDA and/or LPS. In the original studies that characterized this model, we found that EWD enhances NMDA toxicity, but that the release of inflammatory mediators in response to LPS is reduced. The interactions between NMDA and LPS in OHSC during EWD are complex and the impact of natural anti-inflammatory flavonoids, such as rhamnetin, on these responses is currently unknown.

We have previously shown that rhamnetin is anti-inflammatory in LPS-stimulated immortalized BV2 microglia [188]. Thus, we originally predicted that rhamnetin would inhibit LPS-induced proinflammatory mediator release under control conditions and during EWD. The results support both predictions. Rhamnetin reduced LPS elicited TNFalpha and NO release under control conditions (fig. 1) as well as during EWD (fig. 5). Flavonoids have been extensively studied for their anti-inflammatory properties [192] and have been proposed as treatments for neuroinflammatory disorders [155]. The current study supports the potential use of dietary flavonoids such as rhamnetin to reduce neuroinflammation and suggests that they may also be effective in reducing neuroinflammation during EWD.

In addition to evaluating the anti-inflammatory effects of rhamnetin, the current study aimed to assess its potential for reducing excitotoxicity during EWD. We have previously shown that rhamnetin has agonist activity at alpha7 nAChRs [188] and this predicts that the flavonoid should reduce NMDA toxicity both under control conditions

and during EWD. In support, nicotine exposure reduces excitotoxic injury in OHSC under control conditions [189] and during EWD [100] most probably via alpha7 nAChRs [99]. In the current study, rhamnetin reduced NMDA-induced toxicity during EWD (fig. 6), but was inactive under control conditions (fig. 2). The lack of effect under control conditions is surprising because flavonoids are well known for their antioxidant properties [193] and they have been shown to be protective against a variety of excitotoxic insults by reducing oxidative stress [194-196]. However, the neuroprotective effects of rhamnetin during EWD suggest that the mechanism of enhanced excitotoxicity following ethanol exposure is a target for rhamnetin. The specific mechanism targeted remains unknown but these data suggest that dietary flavonoids such as rhamnetin have the potential to reduce excitotoxicity during EWD.

The current study also aimed to evaluate the impact of rhamnetin on the combined effects of NMDA and LPS. Rhamnetin afforded no protection against the combined insults under control conditions (fig. 2) or during EWD (fig. 6). The mechanisms by which rhamnetin reduce inflammatory responses are not certain. Our previous studies on BV2 cells suggest that direct effects via alpha7 nAChR is one component. However, changes in microglial phenotype may play a prominent role here. As mentioned in the introduction, microglia exposed chronically to flavonoids and alpha7 nAChR agonists have been shown to shift towards an anti-inflammatory and neuroprotective phenotype [190, 191]. From our previous studies it was apparent that chronic exposure to ethanol also produced changes in microglia towards a neuroprotective phenotype and similar conclusions have been reached by others using an *in vivo* model [135]. It is therefore likely that the interactions between ethanol, flavonoids, and alpha7 nAChR agonists on microglial phenotype are complex and it will be necessary to explore these in order to explain the results obtained here.

Throughout the current study, 25uM and 100uM rhamnetin were tested. Both concentrations were equipotent at inhibiting LPS-induced inflammatory mediator release under control conditions and during EWD as well as against NMDA toxicity during EWD. However, exposure to 100uM rhamnetin spontaneously induced NO release and toxicity during EWD (fig. 7). The mechanism is unknown but this effect confounds interpretation of results on NO and PI uptake with 100uM rhamnetin during EWD on slices treated with NMDA and/or LPS. This interaction is a potential concern for the proposed use of this class of flavonoids in preventing ethanol-induced neurotoxicity and suggests a potential shift in their therapeutic index following ethanol exposure given the fact that 100uM rhamnetin had no toxic effect under control conditions. However, given the fact that flavonoids are absorbed in the low uM range in animals [170], concentrations as high as 100uM are unlikely to achieved *in vivo* and the lower concentration of 25uM rhamnetin, which was just as anti-inflammatory and neuroprotective as 100uM, had no effect on spontaneous NO release or toxicity. Similar toxicity issues have been reported for quercetin, another flavonoid structurally related to rhamnetin, at high concentrations (40uM) despite being protective at lower concentrations (5uM) against amyloid beta induced toxicity in cultured neurons [197].

In summary, rhamnetin reduced LPS induced inflammation both under control conditions and during EWD, as well as preventing EWD-enhanced excitotoxicity. However, rhamnetin did not reduce excitotoxicity under control conditions and had no effect on the combined effects of NMDA and LPS. Thus, the results support only some of our original predictions. However, the current study shows that rhamnetin (1) retains its anti-inflammatory effects during EWD and (2) specifically targets a mechanism by which ethanol exposure enhances excitotoxicity during EWD. As such, rhamnetin is anti-inflammatory and neuroprotective during EWD. Therefore, rhamnetin and dietary

flavonoids with similar pharmacology have potential value in the treatment of ethanol-induced neurotoxicity.

CHAPTER 5. OVERALL CONCLUSIONS

5.1 Review

Alcohol-induced neurodegeneration has a complex etiology involving several mechanisms of toxicity, including oxidative stress, neuroinflammation and excitotoxicity. All have been extensively studied individually and all are thought to significantly contribute to this pathology. However, in order to develop effective pharmacotherapies against alcohol-induced neurodegeneration, it is now crucial to investigate the interactions between individual mechanisms. Moreover, this suggests the need for multi-functional pharmacotherapies that can reduce all pathological mechanisms simultaneously. Thus, the purpose of the current dissertation was to increase our understanding of the effects of neuroinflammation on excitotoxicity during ethanol withdrawal (EWD) as well as evaluate if novel flavonoids with alpha7 nAChR activity are suitable candidates for reducing ethanol-induced neurotoxicity.

Aim 1: Determine the effects of LPS-induced neuroinflammatory signaling on NMDA-induced toxicity following ethanol exposure in organotypic hippocampal slice cultures (OHSC - Chapter 2)

The primary hypothesis for this study was that ethanol exposure enhances neuroinflammatory signaling which, in turn, potentiates excitotoxicity during EWD. However, despite the fact that neuroinflammatory signaling enhances excitotoxicity under control conditions, the reverse is observed during EWD. This is accompanied by a desensitized inflammatory response following ethanol exposure. Moreover, BV2 microglia exposed to ethanol exhibit an unusual phenotype that may be immunoregulatory. Therefore, the results do not support the hypothesis and instead suggest that, in OHSC, changes to neuroinflammatory signaling induced by ethanol exposure are protective against excitotoxicity during EWD.

Aim 2: Identify and characterize plant natural products with alpha7 nAChR activity (Chapter 3)

The primary hypothesis for this study was that specific flavonoids selectively bind to alpha7 nAChR (relative to alpha4-beta2 nAChR) and this activity enhances their anti-inflammatory properties. Some flavonoids, such as rhamnetin, were found to specifically displace [3H]-MLA whereas others, such as sakuranetin, did not. When comparing rhamnetin and sakuranetin for inhibition of LPS-induced inflammatory mediator release from BV2 microglia, rhamnetin was found to benefit from an alpha7 nAChR mediated effect whereas sakuranetin did not. The results support the original hypothesis and suggest that flavonoids with alpha7 nAChR activity, such as rhamnetin, have the potential to reduce neuroinflammation and excitotoxicity associated with ethanol-induced neurotoxicity.

Aim 3: Evaluate the anti-inflammatory and neuroprotective properties of rhamnetin following ethanol exposure in OHSC (Chapter 4)

The primary hypothesis for this study was that rhamnetin reduces neuroinflammatory signaling and excitotoxicity under control conditions and during EWD. Rhamnetin was found to inhibit neuroinflammatory signaling under control conditions and during EWD, and inhibit excitotoxicity during EWD but not under control conditions. Moreover, rhamnetin had no effect on the combination of neuroinflammatory and excitotoxic insults either under control conditions or during EWD. These results support, in part, the original hypothesis and suggest that rhamnetin has potential value in reducing neuroinflammation and excitotoxicity during EWD.

5.2 Discussion

In the last couple of decades, flavonoids have attracted a lot of attention as potential treatments for neurodegenerative diseases. This has most likely been fueled by the fact that oxidative stress and neuroinflammation are central pathological mechanisms in a great majority of neurodegenerative disorders and as such, flavonoids constitute good drug candidates to reduce both. In fact, flavonoids have been extensively shown to be protective in a variety of preclinical models of neurodegeneration (see below) and this has been generally ascribed to their antioxidant and anti-inflammatory activities. However, flavonoids are not all equally potent neuroprotectants despite having equivalent antioxidant and/or anti-inflammatory properties. This suggests that other pharmacological activities may be important. As such, flavonoids have been shown to directly and indirectly affect a variety of pharmacological targets in the CNS and the work presented herein adds to their rich pharmacology.

Flavonoids modulate GABA receptors with reports dating back to 1983 (reviewed elsewhere [198]) which may explain their well accepted anxiolytic effects [199]. Flavonoids are well characterized cholinesterase inhibitors and are therefore being developed as pharmacotherapies for the treatment of Alzheimer's disease (reviewed elsewhere [200]). Considerable evidence shows that flavonoids have specific effects on the glutamate system and that this contributes to their ability to reduce excitotoxicity (see below). Some reports indicate that they interact with the dopamine transporter [201-203] and this activity may contribute to their neuroprotective effects in preclinical models of Parkinson's disease [204, 205]. Inhibitory effects on the serotonin 5-HT3(A) receptor have also been reported [206]. Therefore, flavonoids constitute a great source of bioactive compounds with a rich structural and pharmacological diversity.

However, this implies that the pharmacology of each specific structure needs to be well characterized depending on their intended use.

The original discovery that rhamnetin has potential alpha7 nAChR agonist activity (chapter 3) implied that in addition to its antioxidant [181] and anti-inflammatory properties [171] this flavonoid would also potentially inhibit excitotoxicity. In fact, flavonoids have been extensively shown to reduce excitotoxicity. However, these studies primarily focus on their direct and indirect effects on the glutamate system and do not suggest nAChR mediated effects. For example, epigallocatechin-3-gallate normalizes glutamate and glycine release from hippocampal and cortical neurons following ischemic injury [207], puerarin inhibits aspartate and glutamate release induced by ischemia in rats [208], myricetin reduces glutamate-induced intracellular Ca^{2+} entry in cultured neurons by inhibiting the phosphorylation of NR1 subunits thus reducing NMDA receptor function [209], apigenin directly inhibits NMDA receptors in cultured cortical neurons [210], and quercetin inhibits glutamate evoked inward currents in xenopus oocytes expressing the human AMPA receptor [211]. This again illustrates their pharmacological diversity and points to potential future lines of investigation to explain the results obtained in chapter 4 (see below).

Before evaluating the neuroprotective effects of rhamnetin, its anti-inflammatory properties were characterized on LPS-stimulated BV2 microglia. We compared the anti-inflammatory effects of rhamnetin and sakuranetin and found that their concentration response curves clearly differed in steepness at the point of inflection of the sigmoidal function. This phenomenon can be calculated when fitting the data using non-linear regression by adding a variable slope parameter to the sigmoidal equation. This parameter is commonly calculated in binding experiments to evaluate cooperative binding of a ligand to a receptor. In binding experiments, this parameter is commonly

known as the Hill coefficient because it was originally defined by Archibald Hill when he discovered the cooperative binding nature of oxygen to hemoglobin. In contrast, in the current study, the sigmoidal function with variable slope is used to fit the effects of a drug on a biological response. Therefore, differences in the steepness of the slope for the anti-inflammatory effects of rhamnetin and sakuranetin suggest that there are differences in their anti-inflammatory mechanisms. Moreover, the fact that this parameter can be manipulated by nAChR antagonists (for rhamnetin) and agonists (for sakuranetin) suggests that the difference resides in their activity at nAChRs. Taken together, these results suggest that rhamnetin benefits from an alpha7 nAChRs mediated anti-inflammatory mechanism whereas sakuranetin does not. In support, similar studies to the ones performed with BV2 microglia (saturation binding and flavonoid anti-inflammatory studies) were undertaken on RAW 264.7 macrophages, a cell line derived from peripheral macrophages, on which we found no evidence of nAChRs, and no differences were observed between rhamnetin and sakuranetin (data not shown). Moreover, closely following the completion of these studies, quercetin (which differs from rhamnetin by a single hydroxyl group) was thoroughly evaluated for its effects on acetylcholine induced inward currents in xenopus oocytes expressing various nAChRs [212-214]. Interestingly, quercetin was found to enhance acetylcholine induced inward currents through alpha7 nAChRs by binding to its Ca^{2+} binding site [153] thus suggesting a positive allosteric mechanism of action. In our studies, given the intrinsic anti-inflammatory activity of rhamnetin, it is difficult to definitively characterize its mechanism of drug action at alpha7 nAChRs. As such, we attempted to further characterize the binding of rhamnetin to alpha7 nAChRs using single molecule fluorescence correlation spectroscopic methods in collaboration with Dr. Chris Richards (University of Kentucky, College of Arts and Sciences, Department of Chemistry). Unfortunately, despite the fact that rhamnetin had the desired fluorescence spectrum to

run these experiments, when diluted at single molecule concentrations, rhamnetin could not be detected. Moreover, ligands with this sort of modest affinity are very difficult to evaluate using this system. Therefore, we were unable to run these experiments. Nevertheless, the BV2 microglia data suggest potential agonist activity for rhamnetin at alpha7 nAChRs and additional studies are required to elucidate its mechanism of drug action.

Before evaluating rhamnetin for its neuroprotective and anti-inflammatory properties, we developed a model of ethanol-induced neurotoxicity *in vitro* which included an excitotoxic and neuroinflammatory component (chapter 2). Current evidence suggests that alcohol dependence ultimately results in neuroinflammation which contributes to alcohol-induced neurodegeneration (see section 1.2.2). However, alcohol dependence is a chronic relapsing disorder and as such, the onset of neuroinflammation is not likely to be sudden. Therefore, the timing and the nature of changes to neuroimmune cells induced by ethanol are likely to be important for disease progression. However, to date, they remain largely unknown. Moreover, it is unclear how these changes interact with other pathological mechanisms during EWD. The results presented in chapter 2 show that, in OHSC, following ethanol exposure, neuroinflammatory processes are protective against NMDA receptor mediated excitotoxicity during EWD (fig. 2.1). Moreover, following ethanol exposure, OHSC exhibit a desensitized response to LPS (fig. 2.2). The specific mechanisms for these effects remain uncertain but results from BV2 microglia exposed to the same paradigm suggest that ethanol polarizes microglia towards an immunoregulatory phenotype [140]. Similarly, in rats exposed to a modified Marjchowicz 4-day ethanol binge, microglia become partially activated and exhibit an anti-inflammatory and neuroprotective phenotype [135]. Thus, collectively these studies suggest that ethanol exposure induces

phenotypic changes in microglia that are reparative and beneficial. It is important to note that, in our studies, ethanol concentration *in vitro* remains above ~40mM throughout exposure [56]. Similarly, in the modified Marjchowicz 4-day ethanol binge, based on the behavioral intoxication scores recorded in these studies [215] it appears that rats are continuously exposed to ethanol. Together, this suggests that polarization of microglia towards an anti-inflammatory and protective phenotype is promoted by *continuous* exposure to ethanol and this phenotype manifests during what could be considered the "first" EWD. As such, the effects of repeated EWD on microglial phenotype are uncertain and may in fact promote a proinflammatory microglial phenotype as has been shown in other ethanol exposure paradigms [39, 40, 216-218]. Given the relapsing nature of alcohol dependence, additional studies are warranted to explore this hypothesis.

One of the limitations of this study is the limited number of neuroinflammatory markers measured. The original hypothesis stated that ethanol exposure would enhance proinflammatory cytokine release which in turn would enhance excitotoxicity. As such, we chose to focus on TNFalpha and NO. When we found that the results did not support the hypothesis, we attempted to measure IL-10 (prototypical anti-inflammatory cytokine) in our culture samples. Unfortunately, IL-10 levels were below the limits of detection in all treatment groups and this may have been due to freeze-thaw degradation of the samples. However, future studies should include additional neuroinflammatory markers in order to get a broader idea of how neuroimmune function is affected. In addition, it would be interesting to evaluate microglial phenotype in the cultures following ethanol exposure and EWD, using e.g. immunohistochemical analyses, which would provide a clearer picture of the changes to this cell type specifically.

With this model of ethanol-induced toxicity at hand, we proceeded to test rhamnetin for its anti-inflammatory and neuroprotective properties. Flavonoids have in

fact already been shown to be cytoprotective in a variety of models of ethanol-induced toxicity. For example, cyanidin-3-glucoside reduces ethanol-induced neurotoxicity in neonatal mice [23] and epigallocatechin prevents alcoholic neuropathy in rats [219]. Moreover, quercetin reverses hepatotoxicity and neurotoxicity induced by 90 days of ethanol administration [22] and prevents alcoholic neuropathy in rats [220]. These studies were undertaken in large part to demonstrate that the antioxidant and anti-inflammatory properties of these compounds translate to neuroprotection against ethanol-induced toxicity. However, in the current study we were interested in evaluating both the anti-inflammatory and the anti-excitotoxic effects of rhamnetin separately and simultaneously. In addition to retaining anti-inflammatory activity during EWD (fig. 4.5), rhamnetin inhibited NMDA toxicity during EWD (fig. 4.6). However, rhamnetin afforded no protection against NMDA alone under control conditions (fig. 4.2). This discrepancy suggests that the mechanism of enhanced excitotoxicity induced by ethanol exposure is a target for rhamnetin. The specific mechanism remains unknown but it is possible that rhamnetin, via nAChRs, normalizes changes to glutamate receptor function induced by ethanol thereby preventing enhanced excitotoxicity during EWD. Indeed, Proctor et al show that the acute effects of ethanol on NMDA receptors are abolished in OHSC from mice chronically exposed to nicotine [221]. Alternatively, given the considerable evidence that flavonoids affect glutamate signaling (see above), it is possible that in addition to its alpha7 nAChR activity, rhamnetin is protective via direct pharmacological effects on the glutamate system. However, additional experiments are needed to test these hypotheses.

In this same study, rhamnetin was found to afford no protection against the combination of NMDA and LPS under control conditions or during EWD (fig. 4.2 & fig. 4.6). Results obtained under control conditions are surprising given the fact that

proinflammatory cytokine release is reduced by rhamnetin and as such is predicted to reduce the potentiation of NMDA toxicity by LPS. However, this assumes that proinflammatory cytokines are responsible for the enhanced toxicity observed under these conditions whereas other mechanisms are possible (discussed in chapter 2). As such, reducing proinflammatory cytokine release does not prevent the potentiating effects of LPS on NMDA toxicity. This either suggests that potentiation of NMDA toxicity by proinflammatory cytokines is governed by an all-or-nothing phenomenon, which implies that high and low concentrations of proinflammatory cytokines are just as effective, or that it is mediated by another mechanism. In fact, several other mechanisms have been proposed for this interaction (discussed in chapter 2) and additional studies are necessary to identify the one responsible. During EWD, the response to LPS was already reduced and rhamnetin further inhibited release of proinflammatory cytokines. However, this did not result in additional protection against NMDA toxicity under these conditions. Chronic exposure to ethanol (see chapter 2), alpha7 nAChR agonists [191], and flavonoids [190] all promote an anti-inflammatory and neuroprotective microglial phenotype. As such, it is impossible to determine how rhamnetin and ethanol co-exposure affect microglial function in these studies and additional studies are necessary to evaluate this interaction. Nevertheless, rhamnetin was found to be anti-inflammatory and reduce excitotoxicity during EWD and therefore constitutes a good candidate to reduce ethanol-induced neurotoxicity.

Interestingly, flavonoids have also been evaluated on ethanol-related behaviors such as voluntary consumption and the anxiogenic effects of EWD. For example, quercetin prevents EWD induced anxiety in mice [222]. Dihydromyricetin [223] and puerarin [224] prevent EWD induced anxiety and reduce voluntary ethanol consumption in rats. Moreover, puerarin was evaluated clinically and found to reduce ethanol intake in

heavy drinkers [225]. Puerarin was originally identified in kudzu root extracts which are used in traditional Chinese medicine as a remedy for hangover. These extracts were also found to be anxiolytic and reduce ethanol consumption in rats [226]. The development of rhamnetin as a potential treatment for ethanol-induced neurotoxicity will require the evaluation of its effects on ethanol-related behaviors such as these in future studies.

The current dissertation stemmed from the discovery of plant extracts with alpha7 nAChR activity and led to the *in vitro* characterization of a novel plant natural product for reducing ethanol-induced toxicity. As such, the work presented herein illustrates the continued value of plants as a source of novel drugs. In fact, almost half of the drugs approved in the last 25 years originate from plants or are derived from plant natural products [227, 228]. This is also true for nAChR drugs [109]. However, although plants are a great source of novel drugs, there are substantial difficulties associated with either the isolation or chemical synthesis of natural products which hinders their commercialization. For example, the development of the anti-cancer drug, paclitaxel (Taxol™), is well known to have suffered from supply problems during preclinical and clinical development [229] as it was exclusively isolated from its original source, Taxus breviflora (Pacific yew). However, using bioengineering strategies, this was eventually circumvented and the current commercial supply of paclitaxel is mainly from plant cell fermentation [230]. As such, plants can be engineered to increase yields of compounds of interest. Moreover, their biosynthetic machinery can be disrupted to stimulate production of novel compounds. Therefore, we developed a strategy that relies on agrobacterium-mediated transformation of *Solidago nemoralis*, for which we obtained proof of transformation (see appendix), to produce a large population of gain-of-function

mutants that potentially have increased yields of the endogenous nAChR active flavonoids and/or novel flavonoid structures.

The end goal of this dissertation was to ascertain the potential value of rhamnetin for the treatment of alcohol-induced neurodegeneration. The various activities of this compound were hypothesized to at least reduce 2 primary pathological mechanisms in ethanol-induced neurotoxicity and we were able to show that, during EWD, rhamnetin is anti-inflammatory and reduces excitotoxicity. Moreover, we developed an *in vitro* model of ethanol-induced neurotoxicity which revealed interesting interactions between neuroinflammatory processes and excitotoxicity during EWD. Given the complex interactions between these pathological mechanisms future research should be aimed at further understanding the progressive changes that occur to neuroimmune cells in alcohol dependence as this may reveal opportune therapeutic windows in the progression of disease for preventing the onset of neurodegeneration.

APPENDIX

HAIRY ROOT CULTURES AND PLANT REGENERATION IN *SOLIDAGO NEMORALIS* TRANSFORMED WITH AGROBACTERIUM RHIZOGENES

5.3 Introduction

Plants have complex biosynthetic machineries that have allowed them to evolve bioactive, complex and multifunctional secondary metabolites as protection against stressors. One example is the complex alkaloid methyllycaconitine (MLA) in Delphinium species which deters herbivorous insects by targeting the insect nicotinic receptor for acetylcholine (nAChR), the most prevalent excitatory receptor in the insect CNS [231]. In addition to being a high affinity ligand for the insect nAChR, MLA is also a highly selective ligand for the alpha7-subtype of human nAChR [111]. Since this receptor is an emerging target for the treatment of neurodegenerative disorders [232] other plant metabolites with this selectivity would be of considerable therapeutic interest. We therefore screened a native plant library for this pharmacological activity using a "differential screening" approach [187] which identified *Solidago nemoralis* ("gray goldenrod") as a prime candidate which has not previously been investigated for this activity. However, as for other plant species, long grow cycles, low yield and necessity to harvest large amounts of biomass, is likely to hinder the development and commercialisation of these metabolites. A number of bioengineering strategies to circumvent these issues have been described for known metabolites with known metabolic pathways [233]. However, in the case of unknown metabolites with unknown pathways for production (as here) an alternative strategy for optimizing plant production is required. The strategy developed in our laboratory relies on Agrobacterium-mediated continuous hairy root culture together with random gain-of-function mutagenesis. Stable mutants over-producing the active metabolites are identified by pharmacological

screening. and are then regenerated into intact mutant plants. However, for the Solidago genus, only one species, S. altissima, has been reported to be transformed by Agrobacterium [234]. Here we report an Agrobacterium-mediated transformation protocol for S. nemoralis, which will enable the application of our genomic optimization strategy to the potential therapeutic compounds contained in this species.

5.4 Materials and Methods

5.4.1 Plant material & culture conditions

Solidago nemoralis seeds were washed with 70% ethanol for 2 min followed by surface sterilization in 30% commercial bleach for 20 min. Seeds were rinsed five times with sterile water and aseptically germinated in plates containing half-strength of Murashigue and Skoog (MS) media supplemented with 0.6% agar and 1% sucrose. For all media, the pH was adjusted to 5.8 and autoclaved. The temperature in the growth chamber was maintained at 25±2ºC with a 12h photoperiod and (light intensity 45 µmol m-2 s-1).

5.4.2 Agrobacterium culture and infection

The pCambia 1301 binary vector was mobilized into Agrobacterium rhizogenes strain R1000 by freeze-thaw method. Briefly, 1 µg of plasmid DNA was mixed with competent cells of A. rhizogenes and incubated on ice for 30 min. The DNA–bacterial mix was frozen in liquid nitrogen for 30 sec and incubated for 5 min in a water bath at 37ºC. After the heat shock, 600 µL of liquid Luria-Bertani (LB) media were added to the bacteria-DNA mix and shaken on rotary shaker set at 200 rpm for 4h at 28ºC. Bacteria were pelleted down by centrifugation at 4000 rpm, resuspended in 100 uL LB and finally plated on solid LB media containing 50 mg/L kanamycin. The plate was incubated for two days at 28ºC which resulted in transformed colonies. A single colony was used to

make transformed bacterial stock which was in turn aliquoted in 1.5mL 60% glycerol suspensions and kept at -80°C.

Five ml of liquid LB kan50 media was inoculated with A. rhizogenes stock harboring pCambia 1301 and grown overnight at 28ºC. Two mL of this culture were used to inoculate 50 mL of LB plus kanamycin 50 mg/L liquid media and grown until the optical density (OD) reached to 0.6. Bacteria were then pelleted down by centrifugation at 4000 rpm and resuspended into 50 mL liquid MS media supplemented with 100 µM acetosyringone (3,5-dimethoxy-4-hydroxy-acetophenone).

Stem explants (4 week old plants), leaf explants (4 week old plants) and root-excised seedlings (2 week old plants) were tested for agrobacterium infection. Leaf explants were excised from stems and stem explants were cut into 1 cm long sections. Explants or seedlings were placed in the agrobacterium culture, wounded using a sterile needle and left to incubate for 20 min. Explants were then blot-dried on sterile filter paper, transferred onto solid MS media supplemented with vitamin B5 and 1% sucrose and incubated for 2 days in the dark in the growth chamber. After the 2-day co-cultivation period, explants were transferred onto MS media plate supplemented with 400 mg/L cefotaxime and 3% sucrose. Hairy roots appeared after 2-3 weeks at which point they were excised from explants, cut into 1 cm long sections and cultured and maintained on MS media supplemented with 250 mg/L cefotaxime and 3% sucrose.

5.4.3 Plant regeneration from hairy roots

All media used in the regeneration process was supplemented with 250 mg/L cefotaxime in order to prevent fortuitous agrobacterium growth. Hairy root cultures were transferred onto MS media alone or MS media supplemented with alpha-naphthaleneacetic acid (NAA) (0.1 or 1 mg/L) or MS media supplemented with NAA (0.1 or 1 mg/L) and 6-benzyladenine (BA) (2 or 5 mg/L). After 4-6 weeks adventitious shoots

appeared. They were excised and transferred onto MS media for root formation. GUS staining was performed at all stages of hairy root culture and plant regeneration to confirm transformation.

5.4.4 beta-glucuronidase (GUS) histochemical assay

GUS staining buffer was prepared by adding 50 mM sodium phosphate (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA and 0.05% Triton X-100 into 150 ml of water. This buffer was dispensed into 15 ml aliquots and stored at –20 ºC. For GUS staining, the stock buffer was thawed and diluted to a final volume of 100 ml in water. 35 mg of 5-bromo,4-chloro,3-indolyl- beta-D-glucuronide (X-Gluc) was dissolved in 150 µl of dimethylformamide and subsequently added to the diluted buffer. Tissue samples were incubated in X-Gluc solution for 24-h at 37 °C and observed for blue staining indicative of GUS expression.

5.5 Results

S. nemoralis stem explants, leaf explants and seedlings were tested for their ability to be transformed by A. rhizogens and produce hairy roots after infection. Leaf explants transformation produced the highest hairy root induction frequency (30-35%) as compared to stem explants (18-20%) and seedlings (6-9%). Therefore, further experiments on regeneration of transformed plants were undertaken with A. rhizogenes transformed leaf explants.

Two-3 weeks after infection, hairy roots appeared on the site of infection. They were excised and cultured independently. When grown in solid MS media without the addition of phytohormones, adventitious shoot regeneration occurred via direct organogenesis. Indeed, once excised, several hairy roots turned green, produced green callus-like structures which ultimately turned into shoots after 4-6 weeks of culture (Fig.

1a,b). However, the frequency of spontaneous shoot regeneration on simple MS media was low (only 2-3 shoots appeared on each hairy root). Therefore, in order to increase shoot regeneration frequency, media was supplemented with either BA/NAA or NAA alone.

A combination of BA (2 or 5 mg/L) and NAA (1 mg/L) did not increase shoot regeneration because hairy roots cultured in this particular medium produced white callus like structures that did not turn into shoot buds (Fig. 1c). However, media with NAA alone (0.1 or 1 mg/L) did produce shoots in 4 weeks of culture (Fig. 1d) after going through both white and green callus like structure stages. Regeneration efficiency was increased by NAA supplemented media which afforded an adventitious shoot budding frequency of ~35%, much higher than in non-supplemented media.

Since the transformation of S. nemoralis was performed with A. rhizogenes strain R1000 harboring pCambia 1301 which has the GUS gene as visible marker, all stages of hairy root development and shoot regeneration were tested by GUS histochemical assay to ensure transformation events (Fig. 2a-c). The coding region of the gusA gene is interrupted by a catalase intron to ensure that the transcription and post-transcription splicing to form mature mRNA occurs by utilizing the plant cell machinery rather than the agrobacterium protein synthesis machinery [235].

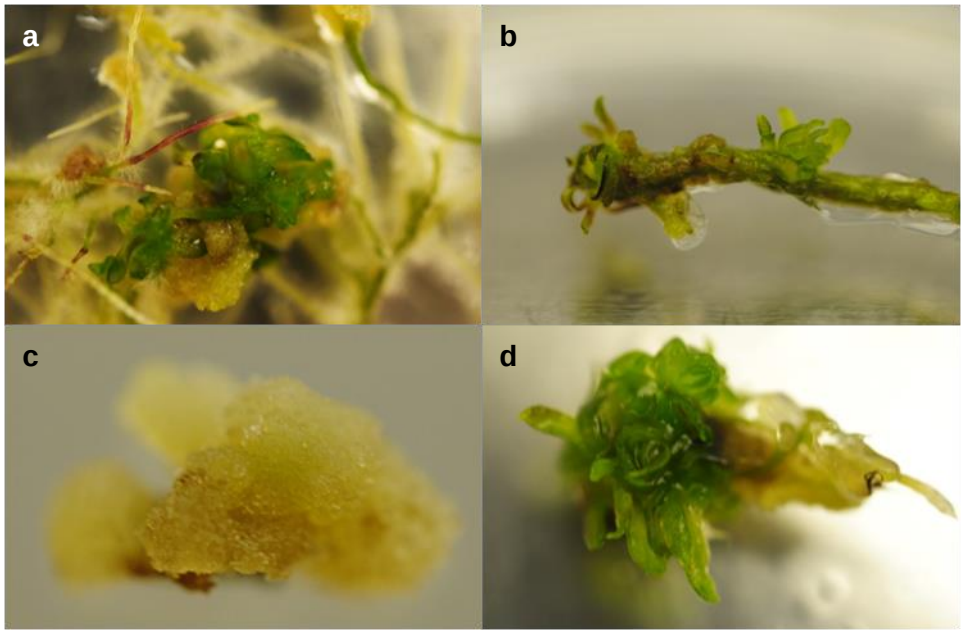

Figure 5.1 Agrobacterium rhizogenes mediated transformation of S. nemoralis

(a) Hairy root culture and their spontaneous regeneration into shoot; (b) Hairy root turned green after two weeks of culture and produced shoots on their surface; (c) Hairy root treated with BA produced white callus which failed to regenerate when continued culture on the same medium; (d) Shoot regeneration increased by application of NAA (0.1 mg/L)

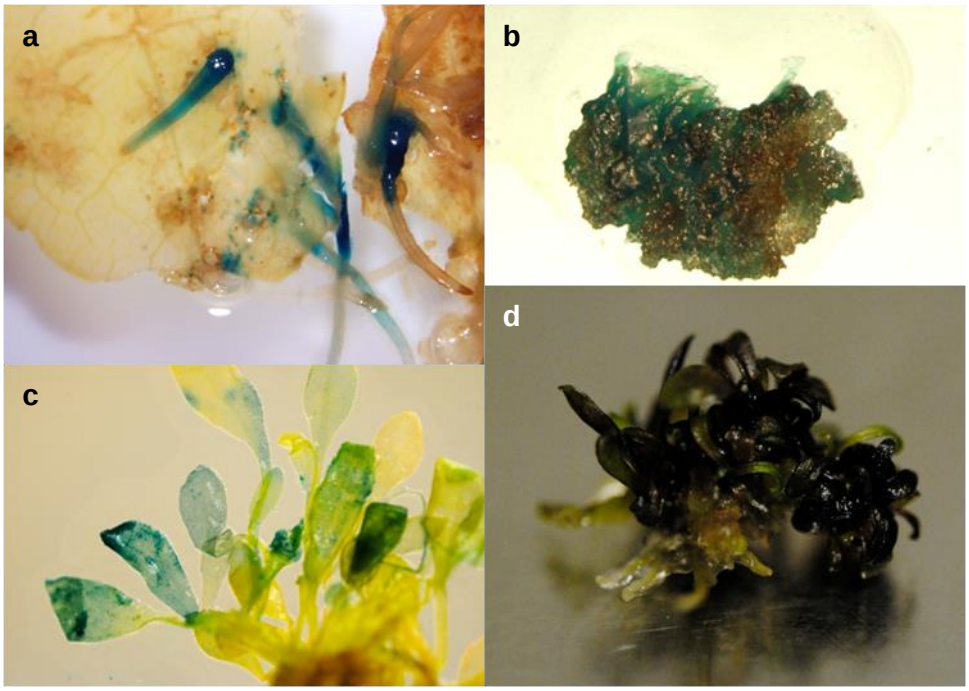

Figure 5.2 GUS histochemical staining of transformed S. nemoralis

Samples were treated in the x-Gluc solution overnight followed by washing with 70% ethanol. (a) Hairy roots generation on the leaf explants; (b) Transformed hairy root turned into callus during regeneration process; (c) Regenerated shoots showed blue GUS stain in their leaves; (d) Adverse effect of high concentration of sucrose in the culture medium- the regenerated shoots turned brown and did not elongate further when HR were cultured on 6% sucrose containing medium.

5.6 Discussion

A. rhizogenes-mediated genetic transformation in plants is a well established procedure which produces hairy roots at the site of infection by altering the endogenous auxin:cytokinin ratio in the plant cell [236, 237]. Additionally, it is well documented that, once established, hairy roots can be cultured on hormone-free medium [238]. S. nemoralis hairy roots were easily established and then cultured both in solid and liquid hormone free medium.

Although shoot regeneration occurred spontaneously in hormone-free media, it occurred only at low frequency. Additionally, introducing exogenous NAA and BA lead to no improvement in shoot regeneration. However, application of NAA alone on S. nemoralis hairy root cultures showed a positive effect on shoot regeneration. These results go against the notion that higher levels of cytokinin (in the auxin:cytokinin ratio) are necessary for shoot regeneration although it has been reported that A. rhizogenes transformed plants can regenerate shoots in the presence of only auxin, presumably due to the cytokinin mimetic effect of the over expression of the rolC gene, present on A. rhizogenes Ri plasmid [239].

The effect of auxin application for shoot regeneration may be different for each hairy root and might be linked with the expression of the rolC gene [240]. A similar result has been previously reported in Solanum khasianum hairy roots where application of IAA and kinetin affected the growth and regeneration capacity of the plant [241].

Additionally, high concentrations of sucrose appear to have an adverse effect on root culture and shoot organogenesis. S. nemoralis hairy root grew better in media containing only 1-2% sucrose. On the other hand, media with more than 4% sucrose turned the root culture and regenerated shoots deep brown in color and abrogated

growth (Fig. 2d). Similar findings on the effect of sucrose concentration on growth and shoot regeneration have previously been reported for Hypericum perforatum [242].

In conclusion, we report a protocol for culture and transformation of S. nemoralis which should enable the genome of this species to be altered so as to optimize production of the potentially valuable pharmacologically active metabolites it contains.

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Joseph Lutz

EDUCATION

Degree

2008-current — Ph.D. in Pharmaceutical Sciences (Drug Discovery), College of Pharmacy, University of Kentucky, Lexington, Kentucky*

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2005 – 2006 — Research assistant, Kentucky Tobacco Research and Development Center (KTRDC), University of Kentucky, Lexington, Kentucky

Project: Natural products genomics – agrobacterium transformation of medicinal plants

PUBLICATIONS

Peer reviewed

2014 — Lutz, J.A., Rogers, D.T., Kulshrestha, M., & Littleton, J.M. (2014). A nicotinic receptor mediated anti-inflammatory effect of the flavonoid rhamnetin in BV2 microglia. Fitoterapia, 98 (October), 11-21

2013 Lutz, J.A., Gunjan, S. K., Bushong, A., Rogers, D. T., & Littleton, J.M. (2013). Hairy Root Cultures and Plant Regeneration in Solidago nemoralis Transformed with Agrobacterium rhizogenes. American Journal of Plant Sciences, 4 (August), 1675–1678

2011 Gunjan, S.K., Rogers, D.T., Czarnecki, J., **Lutz, J.A.**, & Littleton, J.M. (2011). Survival Selection Reveals Altered Pharmacological Phenotypes in *Nicotiana tabacum* var. SR1 Activation Tagged Mutants. Current Trends in Biotechnology and Pharmacy, 5 (1) (January), 1064–1072

Manuscripts in preparation

2014* Lutz, J.A., Carter, M., Fields, L., Barron, S., & Littleton, J.M. Altered relation between lipopolysaccharide-induced inflammatory response and excitotoxicity in rat organotypic hippocampal slice cultures during ethanol withdrawal

2014* Lutz, J.A., Carter, M., Fields, L., Barron, S., & Littleton, J.M. Evaluating the anti-inflammatory and neuroprotective effects of rhamnetin against ethanol-withdrawal induced neurotoxicity in rat organotypic hippocampal slice cultures

* expected submission

HONORS & AWARDS

2011 – 2014 Student Merit Award, Research Society on Alcoholism, NIAAA

PROFESSIONAL PRESENTATIONS

Invited presentations

2013 Evaluating the mechanisms for anti-inflammatory and neuroprotective properties of flavonoids in alcohol neurotoxicity, Pharmacology paper session, Research Society on Alcoholism, Orlando, FL

2011 Flavonoids with putative alpha7 nicotinic receptor activity as potential neuroprotective compounds against alcohol induced neurodegeneration, C.N.S. pharmacology paper session, Research Society on Alcoholism, Atlanta, GA

Poster presentations

2013 Lutz, J.A., Carter, M., Rogers, D.T., Barron, S., Littleton, J.M. Evaluating the mechanisms for anti-inflammatory and neuroprotective properties of flavonoids in alcohol neurotoxicity. Annual Research Society on Alcoholism (RSA) Scientific Meeting, Orlando, FL

2012 Lutz, J. A., Rogers, D.T., D'Souza El-Guindy, N., Littleton, J.M. Anti-inflammatory properties of specific flavonoids is mediated through alpha7 nicotinic receptors: implications for alcohol induced neurodegeneration.

Annual Research Society on Alcoholism (RSA) Scientific Meeting, San Francisco, CA

2011 Lutz, J. A., Rogers, D.T., Kulshrestha, M., Littleton, J.M. Flavonoids with putative alpha7 nicotinic receptor activity as potential protective compounds against alcohol induced neurodegeneration. Annual Research Society on Alcoholism (RSA) Scientific Meeting, Atlanta, GA

2010 Lutz, J. A., Rogers, D.T., Kulshrestha, M., Pauly, J.R., Littleton, J.M. Characterization of a novel binding activity in plant extracts putatively to alpha-7 nicotinic acetylcholine receptors. Society for Neuroscience (SfN) Annual Meeting, San Diego, CA

Campus presentations

2012 An apple a day keeps dementia away: plant flavonoids for alcoholism, Department of Psychology Brown Bag, College of Arts & Science, University of Kentucky, Lexington, KY

2011 Flavonoids with nicotinic receptor activity as multi-functional neuroprotective agents against alcohol induced neurodegeneration, Drug Discovery seminar, College of Pharmacy, University of Kentucky, Lexington, KY

2011 Flavonoids with nicotinic receptor activity as neuroprotective agents, Symposium on Drug Discovery and Development, College of Pharmacy, University of Kentucky, Lexington, KY

2011 Persuading plants to make neuroprotective compounds, Kentucky Tobacco Research & Development Center, University of Kentucky, Lexington, KY

2011 Neuroprotective properties of flavonoids: implications in alcohol induced neurodegeneration, Drug Discovery seminar, College of Pharmacy, University of Kentucky, Lexington, KY

2010 Novel neuroprotective compounds from Kentucky native plants, Drug Discovery seminar, College of Pharmacy, University of Kentucky, Lexington, KY

TEACHING EXPERIENCE

2008 – 2010 Proctor, College of Pharmacy, University of Kentucky, Lexington, KY

2008 – 2009 Teaching assistant, Physiological basis for therapeutics, College of Pharmacy, University of Kentucky, Lexington, KY

UNIVERSITY SERVICES

2008 – 2012 Drug Discovery track representative, American Association of Pharmaceutical Sciences Student chapter, College of Pharmacy, University of Kentucky, Lexington, KY*

* Awarded AAPS student chapter of the year in 2012

2008 – 2011	Student representative for the graduate program committee, College of Pharmacy, University of Kentucky, Lexington, KY
2010	Recruiter for the Pharmaceutical Sciences graduate program at SERMACS (Southeastern Regional Meeting of the American Chemical Society), New Orleans, LO
2011	Recruiter for the Pharmaceutical Sciences graduate program at SERMACS (Southeastern Regional Meeting of the American Chemical Society), Richmond, MA

PROFESSIONAL MEMBERSHIPS

2009 – present	Research Society on Alcoholism
2008 – present	American Association for the Advancement of Science
2009 – present	The Rho Chi Society
2009 – 2012	Society of Neuroscience
2009 – 2012	American Association of Pharmaceutical Sciences
2008 – 2012	Kentucky Academy of Science

SCIENTIFIC OUTREACH

2010 – present	Science fair judge at the Fayette county science fair (2010 – 2013), Central Kentucky science fair (2010), Kentucky State science fair (2010)